Saskatchewan
Agriculture
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AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020032

SIGNIFICANCE AND PATHOGENESIS OF AIDA-1 E. COLI INFECTION IN PIGS

Funded by: The Agriculture Development Fund

March 2007

Prepared by: University of Saskatchewan

SADF FINAL RESEARCH REPORT

PROJECT TITLE:

Significance and pathogenesis of AIDA-I *E. coli* infection in pigs (#20020032)

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SADF FINAL RESEARCH REPORT

Significance and pathogenesis of AIDA-1 *E. coli* infection in pigs (SADF Project # 20020032)

ABSTRACT

Escherichia coli is one of the most common causes of neonatal diarrhea in piglets. To cause diarrhea *E. coli* must attach to the intestinal surface using adhesive factors termed adhesins. AIDA-I is a recently recognized adhesin of *E. coli* isolated from human infants and pigs, but mechanism by which this bacteria cause diarrhea was not known. In this study we demonstrated that this AIDA-I adhesin facilities attachment of *E. coli* to intestinal mucosa, induces production of intestinal biofilm within mucus and is necessary for development of neonatal diarrhea in piglets. Based on these findings, the future studies will be able to develop appropriate vaccine against diarrhea induced by this AIDA-I *E. coli*.

EXECUTIVE SUMMARY:

Escherichia coli is one of the most common causes of neonatal diarrhea in piglets resulting in marked direct and indirect economic losses. To cause diarrhea *E. coli* must attach to the intestinal surface using adhesive factors termed fimbriae and adhesins ("like a boat to a dock"). Our diagnostic laboratory (serving Alberta and Saskatchewan) is experiencing an increase in number of submitted cases of porcine diarrhea associated with *E. coli* bacteria that lack the expression of known attachment factors for pigs. In our previous studies, *E. coli* was isolated from 170 clinical cases of porcine diarrhea and only 25 isolates were positive for known pathogenic attaching factors in pigs (F4 [n=13], F5 [n=7]or EAE [n=5]). Additional 15 isolates were positive for an attachment factor called AIDA-1 (**a**dhesin **i**nvolved in **d**iffuse **a**dherence), the pathogenicity of which has not been determined in porcine diarrhea (Ngeleka et al., 2003; Pritchard et al., 2004). AIDA-1 *E. coli* has been associated with diarrhea in children (Benz and Schmidt, 1989) and our subsequent studies have demonstrated that two *E. coli* isolates positive for AIDA-1 adhesin caused diarrhea in colostrum-deprived piglets (Ngeleka et al., 2003; Pritchard et al., 2004). Little is yet known about the prevalence, significance and pathogenesis of AIDA-1 *E. coli* in people and even less in pigs.

This research project investigated: ***(i)*** prevalence and significance of AIDA-1 *E. coli* in modern swine industry in Alberta and Saskatchewan, ***(ii)*** mechanisms of bacterial attachment to intestinal surface (a crucial step of infection, the understanding of which is necessary for development of preventive measures/vaccines), ***(iii)*** intestinal receptors ("handles") that bacterial adhesion factors ("arms") attach to, and ***(iv)*** age-associated expression of intestinal receptors for AIDA-1 *E. coli*.

The following activities focused on characterization of AIDA-1 adhesin isolated from porcine AIDA-1 strains were completed: ***i)*** AIDA-1 adhesin was isolated and purified from porcine AIDA-1 *E. coli*, ***ii)*** identity of isolated protein was confirmed by partial amino acid sequencing of porcine AIDA-1 adhesin, ***iii)*** anti-AIDA polyclonal antibody was obtained by immunization of rabbits and ***iv)*** comparative detection by co-agglutination and immuno-dot blot of AIDA-1 positive and negative *E. coli* isolates were performed. We demonstrated that AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* clinical isolates is an acidic protein consisting of five isoforms. It has a molecular weight (100 kDa) similar to the AIDA-I adhesin expressed by human AIDA-I positive *E. coli* strain 2787 and has a relatively high amino acid homology (78-87%) with it

(Fang et al., 2005). A manuscript describing these findings has been published in Veterinary Microbiology (see attached reprint).

Investigation of the role of AIDA-1 adhesin in attachment to intestinal mucosa and pathogenesis of diarrhea was very productive. This investigation was conducted *in vitro* and *in vivo* by using the following bacterial strains: i) wild type strain: AIDA-I$^+$/STb$^+$ *E. coli* isolated from a clinical case of porcine diarrhea, ii) mutant strain: AIDA-I$^-$/STb$^+$ *E. coli*, generated by partial deletion of the *aidA* gene from the wild strain, and iii) complemented strain: AIDA-I+/STb+ *E. coli*, generated by reintroducing the full length *aidA* gene into the mutant strain. Our *in vitro* assays showed marked diffuse adherence to HeLa cells, enhanced bacterial autoaggregation and significant biofilm formation by AIDA-I positive strains, when compared to AIDA-I negative strains. Subsequently, we demonstrated *in vivo* that colostrum-deprived neonatal piglets infected with wild type and complemented strains developed diarrhea 20 and 30 h after oral inoculation, respectively, in contrast to pigs infected with AIDA-I negative strains that did not developed diarrhea. Intestinal colonization was evaluated by histology, imunohistochemistry, transmission electron microscopy, including immunogold electron microscopy, and showed heavy bacterial colonization with biofilm formation in the large intestine with AIDA-I positive strains, but not in pigs infected with AIDA-I negative strains. We demonstrated that AIDA-I adhesin is a significant virulence factor for intestinal colonization, induction of biofilm formation and development of diarrhea (Ravi et al., 2007). These findings were published in Veterinary Microbiology (see attached reprint).

Our investigation and characterization of AIDA-1 receptor(s) in porcine intestinal mucus was also very successful. Based on the intimate interaction between AIDA-I$^+$ *E. coli* and mucus within the intestinal biofilm observed in the above-described study (Ravi et al., 2007), we hypothesized that porcine intestinal mucus contains receptor(s) for AIDA-I adhesin. Using affinity chromatography and *in vitro* adhesion assays, we demonstrated that porcine mucus contains 65 and 120 kDa proteins (p65 and p120) that bind with high affinity to purified AIDA-I adhesin and that AIDA-I positive *E. coli* binds to these proteins with higher affinity than do AIDA-I negative mutant and F4 positive *E. coli*. The identity of p65 was not determined based on LCMS/MS data, whereas p120 was matched to two nuclear proteins (namely, DNA damage binding protein and splicing factor 3b) and one cytoplasmic protein, which is an IgG Fc binding protein. Based on amino acid homology, similar molecular weight (Knutton et al., 1991), structural similarity to mucin (Harada et al., 1997) and evidence of being secreted by goblet cells into the intestinal lumen (Knutton et al., 1991), we think that the IgG Fc binding protein is most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin (Fang et al., 2007). A manuscript describing AIDA-I receptors in porcine intestinal mucus was accepted for publication with minor revisions in Veterinary Microbiology.

Due to unsuccessful development of polyclonal antibodies against the above-described p65 and p120 AIDA-I receptors, we failed to determine the age-associated expression of intestinal receptors for AIDA-1 *E. coli*. As we originally proposed, rabbits were immunized with p65 and p120 according to previously reported procedure used successfully many times in our laboratory for various other proteins including AIDA-I adhesin (Fang et al., 2005; Shahriar et al., 2006a; Shahriar et al., 2006b). Nevertheless, antibody titers developed against these proteins by two different rabbits were inadequate for any immunodetection technique even after multiple boost injections that used up the entire sample with isolated AIDA-I receptors. It is possible that immunogenicity of these proteins is very low, therefore, together with commercial TiterMax Gold adjuvant they did not initiate appropriate immunological response in rabbits. In retrospect, we should have tried to use Freund's adjuvant, even though their use is discouraged by both the

University of Saskatchewan Animal Care Committee and Canadian Council on Animal Care. Despite difficulties encountered in this part of our investigation, we hope to continue with this project. We are in the process of requesting antibodies against human IgG Fc binding protein reported by Harada et al. If there is a cross reactivity between human and porcine IgG Fc binding proteins, that we suspect serve as AIDA-I receptor in porcine intestinal mucus based on the above-indicated study, we will be able to determine the age-associated expression of IgG Fc binding protein by porcine intestinal mucosa. In addition to that, we will be able to employ competitive inhibition binding assays in vitro to determine further if IgG Fc binding protein is genuine AIDA-I receptor.

To investigate prevalence of AIDA-1 *E. coli* infection in modern swine industry, we have established collaboration with Gallant Custom Laboratories Inc., Cambridge, Ontario, that provides microbiological services to swine practitioners in Alberta, Saskatchewan and Manitoba. Our team met with the major swine practitioners in the Prairie Provinces at the annual meeting of Western Canadian Association of Swine Practitioners in October, 2004, and discussed/explored the best approach to determine clinical significance and prevalence of AIDA-I *E. coli* infection. A total of 110 *E. coli*, isolated from problematic cases of F4-negative *E. coli* diarrhea in pigs which tested negative for F4 fimbria by standard slide agglutination test, were used in this study. These cases originated from commercial swine herds located in the Western Canadian prairie provinces: Saskatchewan, Manitoba and Alberta, and were submitted to Gallant Custom Laboratories, Ontario, and Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan between 1999-2005. The clinical data for these isolates were obtained by contacting the practitioners and asking them to fill out a questionnaire regarding clinical presentation and all 110 *E. coli* isolates were tested by M-PCR for various known virulence factors of *E. coli*. Nine (8.2%) isolates of 110 F4 negative *E. coli* associated with clinical diarrhea were positive for AIDA-I adhesin. The prevalence of other fimbrial adhesins in the decreasing order include: 1.8% each for F41, F18 and F5, and 0.9% for F6. The prevalence of other non-fimbrial adhesins in the present study include: 8.2% AIDA-I and 3.6% for EAE. 22.7% of isolates carried at least one toxin and 13.6% carried at least one of the adhesins and toxins. 61.8% of the *E. coli* were negative for all tested common adhesin and toxin genes. Clinical information was available for 35 isolates (5 AIDA-I$^+$ and 30 AIDA-I$^-$) representing 18 diarrhea cases. At the farm level, the clinical problem of diarrhea among these 18 cases was persistent (50%), recurrent (22.2%), at single point of time (16.6%) or sporadic (11.1%). These cases represented three different age (in days) groups: 0-7 d (n=9), 8-14 d (n=4) and 21-60 d (n=5), in farrow-finisher swine operations. There were 5 AIDA-I$^+$ *E. coli* isolates representing 3 (16.7%) clinical cases. All the AIDA-I$^+$ *E. coli* belonged to the 21-60 days old group while 87% of the AIDA-I$^-$ *E. coli* were from the pigs younger than 14 days old.

In conclusion, AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* clinical isolates is an acidic 100 kDa protein consisting of five isoforms, which has relatively high amino acid homology with AIDA-I adhesin isolated from human AIDA-I positive *E. coli* isolates (Fang et al., 2005). Using various AIDA-I *E. coli* strains (wild positive, mutant negative, complemented positive) we demonstrated that AIDA-I adhesin is necessary for diffuse adherence to HeLa cells, enhancement of bacterial autoaggregation and biofilm formation *in vitro*. In addition, we demonstrated *in vivo* that AIDA-I adhesin is a significant virulence factor necessary for intestinal colonization, induction of biofilm formation and development of diarrhea (Ravi et al., 2007). Based on intimate interaction between AIDA-I positive *E. coli* and mucus we hypothesized and subsequently demonstrated that porcine intestinal mucus contains AIDA-I receptors (p65 and p120). The identity of p65 was not determined based on LCMS/MS data, whereas p120 was matched to IgG Fc binding protein which is hypothesized to serve as AIDA-I

receptor in porcine mucus (Fang et al., 2007). Based on our *in vivo* experiments and based on survey results, we suspect that AIDA-1 positive *E. coli* infection may interfere with optimal growth of neonatal and postweaning piglets and cause economical loses in modern swine industry primarily due to diarrhea. However, mortality does not seem to be a clinical feature in uncomplicated neonatal infections by AIDA-I positive *E. coli*. Based on limited survey data, it seems that AIDA-I positive E. coli is more common in weaned piglets. If we obtain anti IgG Fc binding protein antibody from Harada et al we will be able to determine if AIDA-I receptors are more abundant in weaned than in suckling piglets. We will also be able to demonstrate co-localization of AIDA-I adhesin and IgG Fc binding protein *in vivo* using immuno-gold electron microscopy. In addition, it would be beneficial to characterize experimental challenge of weaned piglets with AIDA-I *E. coli*, since in this study we used only neonatal piglets. Our future studies will also determine if colostral anti-AIDA-I antibody can prevent diarrhea induced AIDA-I *E. coli* in neonatal piglets and if vaccination using AIDA-I antigens can prevent disease in weaned pigs.

TECHNICAL REPORT

Research team

- Dorothy M. Middleton, BVM&S, PhD, MRCVS, Dipl. ACVP, co-principal investigator, Department of Veterinary Pathology, WCVM, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4; Phone: (306) 966-7298, Fax: (306) 966-7439, e-mail: dorothy.middleton @usask.ca. Dr. Middleton is an ACVP board certified pathologist experienced in mucosal immunity and pathogenesis of porcine infectious diseases. She is experienced in various immunochemical and morphological investigative techniques. Dr. Middleton was the department head loaded with intensive and exhausting administrative duties from July 1, 2002 to June 30, 2005. After that Dr. Middleton was on her administrative and sabbatical leaves, during which she contributed significant amount of her time towards this research program.
- Elemir Simko, DVM, DVSc, Dipl. ACVP, principal co-investigator, Department of Veterinary Pathology, WCVM, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4; Phone: (306) 966-7307, Fax: (306) 966-7439, e-mail: elemir.simko@usask.ca. Dr. Simko is an ACVP board certified pathologist experienced in innate immunity and resistance to infectious diseases. His lab uses various protein biochemistry techniques to isolate and characterize proteins involved in host defense and host pathogen interaction. During the 2004/2005 academic year, he was on sabbatical leave and after that he assumed relatively heavy teaching load in the department.
- Musangu Ngeleka DVM, MSc, PhD, collaborator, Head of Bacteriology, Prairie Diagnostic Services, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4, Phone: (306) 966-7250 Fax: (306) 966-2488, e-mail: musangu.ngeleka@usask.ca. Dr. Ngeleka is a veterinary bacteriologist working on pathogenesis of *E. coli* in domestic animals (pigs, poultry, and recently in calves) for many years. His past experience includes characterization of virulence factors and evaluation of disease development process, mutant development and genetically engineered live vaccine production.
- Madhu Ravi, DVM, MSc, was a full-time graduate student enrolled in MSc program from August 2003 to June 2006, when he defended his MSc thesis entitled 'Pathogenesis and clinical significance of AIDA-I *E. coli* in diarrhea of pigs'. Currently, Dr. Ravi is enrolled in

anatomic pathology residency program to acquired additional diagnostic skills. His research and diagnostic skills acquired in our department are in high demand not only in Saskatchewan and Western Canada, but also in the entire North America. Dr. Ravi's personal financial support was in-part obtained from this funding

- Yuanmu Fang DVM, MSc, was a full-time research technician in our research laboratory. In May 2004 he started his MSc program focused on characterization of porcine AIDA-1 adhesin and its receptor(s) in intestinal mucosa. In April 2007, he will be defending his MSc thesis. Currently, he is employed as a junior researcher in a biotech industry field in Western Canada. Dr. Fang's personal financial support was obtained from this funding.

Rationale and objectives

Escherichia coli is one of the most common causes of neonatal diarrhea in piglets resulting in marked direct and indirect economic losses. To cause diarrhea *E. coli* must attach to the intestinal surface using adhesive factors termed fimbriae and adhesins ("like a boat to a dock"). Our diagnostic laboratory (serving Alberta and Saskatchewan) is experiencing an increase in number of submitted cases of porcine diarrhea associated with *E. coli* bacteria that lack the expression of known attachment factors for pigs. In our previous studies, *E. coli* was isolated from 170 clinical cases of porcine diarrhea and only 25 isolates were positive for known pathogenic attaching factors in pigs (F4 [n=13], F5 [n=7]or EAE [n=5]). Additional 15 isolates were positive for an attachment factor called AIDA-1 (**a**dhesin **i**nvolved in **d**iffuse **a**dherence), the pathogenicity of which has not been determined in porcine diarrhea (Ngeleka et al., 2003; Pritchard et al., 2004). AIDA-1 *E. coli* has been associated with diarrhea in children (Benz and Schmidt, 1989) and our subsequent studies have demonstrated that two *E. coli* isolates positive for AIDA-1 adhesin caused diarrhea in colostrum-deprived piglets (Ngeleka et al., 2003; Pritchard et al., 2004). Little is yet known about the prevalence, significance and pathogenesis of AIDA-1 *E. coli* in people and even less in pigs.

This research project investigated: ***(i)*** prevalence and significance of AIDA-1 *E. coli* in modern swine industry in Alberta and Saskatchewan, ***(ii)*** mechanisms of bacterial attachment to intestinal surface (a crucial step of infection, the understanding of which is necessary for development of preventive measures/vaccines), ***(iii)*** intestinal receptors ("handles") that bacterial adhesion factors ("arms") attach to, and ***(iv)*** age-associated expression of intestinal receptors for AIDA-1 *E. coli*.

Funding

In addition to the funding provided by Saskatchewan Agriculture Development Fund, the principal co-investigators successfully attracted additional funding from the Alberta Agriculture Research Institute ($75,000) and from Sask Pork ($15,000). On top of this funding, a graduate student stipend ($30,000) was secured from the WCVM Interprovincial Graduate Student Fund to compensate the difference between originally expected and subsequently received funding from the Sask Pork. As indicated later, the conceptual idea and purpose has not been changed; however, the research program has been enhanced with a project that contributed to our understanding of prevalence and clinical significance of AIDA-1 *E. coli* infection in modern swine industry in Saskatchewan and Alberta. Together with our collaborator Dr. Musangu Ngeleka, an additional funding ($15,000) was obtained to support in-part investigation of the role of AIDA-I gene and its product in pathogenesis of experimentally induced diarrhea in neonatal piglets using the following bacterial strains: i) wild type strain: AIDA-I$^+$/STb$^-$ *E. coli*

isolated from a clinical case of porcine diarrhea, ii) mutant strain: AIDA-I$^-$/STb$^+$ *E. coli*, generated by partial deletion of the *aidA* gene from the wild strain, and iii) complemented strain: AIDA-I+/STb+ *E. coli*, generated by reintroducing the full length *aidA* gene into the mutant strain. For each dollar obtained from the Saskatchewan Agriculture Development Fund more than 1.25 dollars were acquired from other sources for support of this research program investigating pathogenesis and clinical relevance of intestinal AIDA-I *E. coli* infection in the modern swine production.

Self-evaluation of achievements and challenges

Funding:

We are very pleased with our success in securing additional funding and expanding and enhancing this research program.

Graduate students:

During the first 12 months we encountered difficulties with recruitment of a suitable PhD graduate student for this project. On three individual occasions we had a graduate student interested in this project and formal process of academic acceptance was initiated in two of these cases. Unfortunately, due to various reasons (e.g. family, professional) these students did not come to the University of Saskatchewan. Finally, in August 2003, Dr. Madhu Ravi, an MSc graduate student, joined our research group and completed investigation focused on pathogenesis and clinical significance of AIDA-I *E. coli* in neonatal porcine diarrhea. In addition, Dr. Yuanmu Fang, a former research technician in our laboratory, started his MSc program focused on characterization of porcine AIDA-1 adhesin and its receptor(s) in intestinal mucosa. His research has been also completed.

Principal co-investigators:

Intensive and exhausting administrative duties of Dr. Middleton as well as 12-month sabbatical leave (in Italy) of Dr. Simko had an indirect negative effects on progress of this research, even though great efforts were made to minimize as much as possible this negative impact.

Research:

We are very pleased with our success in this research program. ***Our experiments generated substantial amount of important clinical and pathological data summarized in two published and one accepted papers (see attached) in high quality research journals. The forth paper is being prepared for publication and further studies are being planned. In addition to these publications, both our graduate students were awarded for high quality of their research presented at national and international conferences. Dr. Yuanmu Fang received an award of The Best Graduate Student Research Presentation at the 5th Annual Meeting of the Canadian Animal Health Laboratorians Network, Ottawa, Ontario, 2006, and Dr. Madhu Ravi received the 2nd Prize, Young Investigator Award for Experimental Diseases at 57th Annual Meeting of the American College of Veterinary Pathologists, Tucson, Arizona, 2006.*** Publication of our research in high quality peer-reviewed journals together with the above-mentioned awards received at national and international conferences testify to the high quality of our research supported by the SADF, that was always appropriately acknowledged and promoted.

COMPLETED RESEARCH ACTIVITIES

1. Isolation of AIDA-1 adhesin and development of anti-AIDA antibodies

Materials and Methods:

Isolation of AIDA-1 protein from two of our porcine isolates (PD58 and PD20) was performed according to a previously published method (Stirm et al., 1967) with a few modifications. Briefly, bacteria were cultured in 4L trypticase-soy-broth (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company, Sparks, MD, USA) for 36 hr at 37°C in an incubator shaker and harvested by centrifugation at 6,000 x g for 15 min. Bacterial pellets were washed twice and re-suspended in 100 mL phosphate-buffered saline (PBS)) (0.01M sodium phosphate, 0.15M NaCl, pH 7.2). Surface bacterial proteins were detached by heating to 60°C for 30 min followed by 10 min of friction created by a homogenizer (Polytron PT3000, Kinematica Ag, Littau, Switzerland) using a 5 mm diameter rotor (Homogenizer Generator PT-DA 3007/2) at 10,000 rpm. Bacteria were removed from suspension by centrifugation at 14,000 x g for 15 min. To prevent bacterial proliferation, 0.05% sodium azide was added to the supernatant which was stored at 4°C for 3 day. Naturally precipitated proteins were removed from suspension by suction filtration through 0.45 µm filter membrane (FP-450 PVDF Membrane Filter, Pall Gelman, Ann Arobor, MI, USA). Precipitated proteins were washed from the filter membrane with 50 mL PBS, sedimented by centrifugation at 2000 x g for 15 min at 4°C and re-suspended in 50 mL PBS, pH 7.2. Sedimentation and re-suspension procedure was repeated 3 times. The final step of purification was the concentration of the re-suspended proteins using 30 kDa centrifugal concentrators (30K Filtron Brand Macrosep, Pall Gelman, Ann Arobor, MI, USA). Purity of isolated AIDA-1 proteins was examined by Coomassie-stained sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gels.

Isolated AIDA-1 protein was subjected to one-dimensional SDS PAGE and separated proteins in gel were visualized by Coomassie Brilliant Blue stain. Narrow strips of polyacrilamide gels containing 100 kDa protein bands were excised, placed in PBS (1:1 weight/volume) and homogenized by tissue grinder. The homogenate was emulsified with an equal volume of either Complete Freund Adjuvant (CFA) or Incomplete Freund Adjuvant (IFA) (Sigma Chemical Co, St Louis, MO, USA). The resultant vaccines contained approximately 0.2 mg/mL of AIDA-1 protein. Priming immunization of two rabbits was performed with 1 mL (4x0.25) of the CFA vaccine administered subcutaneously to each rabbit at 4 different sites (0.25 mL per site). Booster immunization with the IFA vaccine was performed at day 28 and day 42 after priming immunization and serum was harvested at day 49 after terminal exsanguination of the rabbits *via* the carotid artery. Serum collected from rabbits before immunization was used as negative control serum

Results:

An approximately 100kDa protein was isolated (Figure 1, Lanes 3 and 4) from a crude suspension of surface proteins of the AIDA-1 positive *E. coli* porcine isolates PD58 and PD20 (Figure 1, Lanes 1 and 2) and detected by Western blot using polyclonal anti-AIDA-1 serum (Figure 1, Lane 5). Two-dimensional SDS PAGE revealed that isolated AIDA-1 protein consists of at least 5 isoforms with isoelectric points ranging from 4.0 to 5.0 (Figure 2).

Conclusion:

AIDA-1 adhesin isolated from porcine *E. coli* isolates has the same molecular weight as the AIDA-1 adhesin of human AIDA-1 positive *E. coli* strain 2787 (Benz and Schmidt, 1989), and a very high amino acid sequence homology. Porcine AIDA-1 adhesin is a relatively acidic protein with at least 5 isoforms, the isoelectric point of which ranges from 4 to 5. The isoelectric point of human AIDA-1 adhesin has not been reported. Given the relatively high degree of homology between amino acid sequences of certain corresponding segments of porcine and human AIDA-1 proteins, we suspect that amino acid sequence homology of both entire AIDA-1 proteins is high,

and that AIDA-1 protein from human *E. coli* isolates is also acidic (Fang et al., 2005). (See attached published manuscript.)

2. *Comparison of porcine and human AIDA-1 adhesins*

Material and Methods:

Isolated AIDA-1 protein was separated by 1D SDS PAGE and visualized by Coomasie Brilliant Blue staining. Protein corresponding to the 100 kDa band was excised from the gel and submitted for identification by liquid chromatography mass spectrometry (LCMS/MS) performed by the UVic-Genome BC Proteomics Centre (University of Victoria, Canada) using QSTAR® XL Hybrid LC/MS/MS System (Applied Biosystems, Foster City, CA, USA).

Results:

Amino acid sequences determined for various fragments of AIDA-1 protein from both PD58 and PD20 isolates had 86.7% and 78.4%, respectively, homology with AIDA-1 protein isolated from human *E. coli* 2787 (Benz and Schmidt, 1992) (Tables 1 and 2). Only one amino acid segment (825 – 836) of the AIDA-1 protein isolated from both PD58 and PD20 was sequenced and in this single segment there was a 100% homology between the AIDA-1 proteins isolated from two different porcine AIDA-1 *E. coli* isolates (Table 3). Despite the relatively high amino acid homology between AIDA-1 proteins expressed by porcine and human *E. coli* isolates, the human AIDA-1 positive *E. coli* strain 2787 was not detected by immuno-dot-blot (Figure 3) and only weakly detected by Western blot (Figure 4, Lanes 3 and 6) using rabbit polyclonal antiserum raised against AIDA-1 protein isolated from porcine AIDA-1 positive *E. coli*. To characterize further these detection differences between human and porcine AIDA-1 positive *E. coli*, we subjected both human and porcine AIDA-1 positive isolates to the same culture conditions and isolation procedures of surface proteins (as described in the Isolation Materials and Methods #1). By rough estimation of staining intensity of crude surface proteins separated by SDS PAGE and visualized by Coomassie Brilliant Blue we determined that a higher amount of a 100 kDa protein, previously determined as AIDA-1 adhesin, was harvested from the porcine PD20 isolate than from the human *E. coli* strain 2787 (Figure 5).

Conclusion:

Based on a limited comparison, it appears that there is a very high homology between AIDA-1 proteins expressed by porcine AIDA-1 positive *E. coli* isolates. We were surprised by a failure to detect human AIDA-1 positive *E. coli* strain 2787 by immuno-dot-blot, and by only a weak detection of human AIDA-1 protein by Western blot using rabbit polycolonal antiserum raised against AIDA-1 protein isolated from porcine AIDA-1 positive *E. coli*. Decreased sensitivity of detection could be explained, at least partially, by an apparent lower AIDA-1 protein production by the human AIDA-1 *E. coli* strain 2787 when compared to the porcine isolate PD20. Potential immunogenic structural differences between AIDA-1 proteins produced by human and porcine *E. coli* strains might also explain failure or weak detection of human AIDA-1 *E. coli* strain 2787 by antibodies raised against porcine AIDA-1 protein in this study. Since antibodies raised against AIDA-1 protein from human *E. coli* were able to detect porcine AIDA-1 positive *E. coli* isolates (Mainil et al., 2002) and since we demonstrated in this study that there is a relatively high amino acid sequence homology between human and porcine AIDA-1 proteins, we suspect that low expression intensity of AIDA-1 protein, rather than a substantial immunogenic variability, was responsible for failure to detect human AIDA-1 *E. coli* strain 2787 by our immuno-dot-blot test (Fang et al., 2005). (See attached published manuscript.)

3. *Detection of expression of AIDA-1 protein*

Materials and Methods:

Immuno-dot-blot

Immuno-dot-blotting was used to detect AIDA-1 adhesin on bacterial surfaces of 17 isolates of *E. coli* determined to be positive for AIDA-1 gene by PCR in our previous study (Ngeleka et al., 2003). Nineteen isolates of various different *E. coli* virotypes negative for AIDA-1 were used as negative controls. Briefly, bacteria were cultured in 5 mL of TSB (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company, Sparks, MD, USA) at 37°C for 48 hours and harvested by centrifugation at 2000 x g for 15 min. Bacterial pellets were re-suspended and washed twice with PBS by repeating centrifugation procedure. The bacterial concentration of each bacterial isolate was adjusted to approximately $2x10^6$ CFUs/mL using previously reported conversion formula for optical density and CFU for *E. coli* ($CFU/mL=OD_{620}x2.5x10^8$) (Steinberg and Lehrer, 1997). Duplicates of 50 µL of suspension of each isolate were dot-blotted (Bio-Dot SF Microfiltration Apparatus, Bio-Rad Laboratories, Mississauga, ON, Canada) to a PVDF membrane using a vacuum generated by running tap water. AIDA-1 positive *E. coli* blotted on the PVDF membrane were detected by chemiluminescence immuno-detection using Bio-Rad reagents and blotting system as described for Western blot. Based on subjective evaluation of intensity of detected chemiluminesence, tested bacterial isolates were determined to have a negative (-), negative with slight positivity (+/-), or various degrees of a positive (+, ++, +++, or ++++) reaction with polyclonal anti-AIDA-1 serum. Both negative and negative with slight positivity were considered as negative reactions in this study.

Coagglutination

AIDA-I positive and negative porcine *E.coli* isolates were cultured in 10 mL of Todd-Hewitt broth at 37°C for 18 hours. After washing with PBS, bacteria were sedimented by 2000 x g centrifugation and re-suspended in 0.5 mL of PBS containing 0.5% formaldehyde. One drop of bacterial suspension was mixed with one drop of positive or negative monovalent coagglutination reagent (CR) (positive CR contained rabbit polyclonal anti-AIDA-1 serum and negative CR contained pre-immunization serum from the same rabbit) prepared as described previously (Gottschalk et al., 1993). After 30 seconds of continuous gentle tilting of slides, results were recorded semi-quantitatively as negative (-), negative with occasional aggregates (+/-), or various degrees of positive reaction (+, ++, +++, or ++++). Both negative and negative with occasional aggregates were considered as negative reactions in this study.

Results:

Using rabbit anti-porcine-AIDA-1 polyclonal serum, we were able to detect AIDA-1 protein on the bacterial surface in 13, 12, and 9 out of 17 PCR-positive AIDA-1 *E. coli* isolates by immuno-dot-blot, co-agglutination, and both immuno-dot blot and coagglutination, respectively; whereas, a positive reaction was detected by immuno-dot-blot and co-agglutination in 2 and 3 isolates, respectively, out of 18 *E. coli* isolates determined to be negative for AIDA-1 gene by PCR (Figure 6 and Table 4). Accordingly, if all *E. coli* isolates determined to contain AIDA-1 gene by PCR also expressed AIDA-1 protein, then the sensitivity of detection of AIDA-1 protein on bacterial surfaces by immuno-dot-blot and co-agglutination was 76 and 71%, respectively, whereas specificity was 89% and 84%, respectively.

Conclusion:

In this study, the sensitivity of detection of surface AIDA-1 adhesin of PCR-positive AIDA-1 *E. coli* by immuno-dot-blot and coagglutination tests was 76 and 71%, respectively, whereas specificity was 89% and 84%, respectively. Mainil et al. (2002) achieved higher sensitivity (93%) and specificity (90%) of detection of AIDA-1 protein in porcine *E. coli* isolates by Western blot using immune serum that was raised against a recombinant *E. coli* K12 (pIB4) expressing AIDA-1 protein cloned from the human *E. coli* strain 2787 and preabsorbed with *E. coli* K12 (pBR322). Even

though coagglutination detection of AIDA-1 positive *E. coli* is a very simple and fast test it is unlikely to be used routinely for diagnostic detection of AIDA-1 positive *E. coli* due to relatively low sensitivity. Specificity of immuno-dot-blot and coagglutination detection of AIDA-1 adhesin on the bacterial surface is relatively high and these tests are potentially useful for identification of false positive reactions by other detection methods (Fang et al., 2005). (See attached published manuscript.)

4. Pathogenesis of AIDA-1 E. coli attachment to intestinal surface

Investigation of significance of AIDA-1 adhesin in bacterial attachment to intestinal mucosa as well as pathogenesis of diarrhea caused by AIDA-1 *E. coli* were carried out *in vitro* and *in vivo* with a lot of success.

Pathogenesis of AIDA-1 *E. coli* attachment to cells *in vitro*

Role of AIDA-1 adhesin in bacterial attachment and biofilm formation *in vitro*

We demonstrated that AIDA-1 adhesin of porcine AIDA-1 *E. coli* mediates adhesion to HeLa cells (biotic or "living" surface) by comparative investigation of the attachment to HeLa cells of AIDA-1 positive (wild type isolated for diarrheic piglets), the AIDA-1 negative mutant *E. coli* and the AIDA-1 positive complemented mutant *E.coli* (AIDA-1 gene reinserted) (Figure 7). HeLa cell adhesion assay was adapted from Benz and Schmidt (Benz and Schmidt, 1989). Attachment to and biofilm formation on abiotic (non-living) surface (polysterene) was examined by phase-contrast microscopy (Danese et al., 2000) (Fig. 8). Biofilm formation was also quantified by a colormetric method adapted from Wakimoto *et al. (Wakimoto et al., 2004)*. The ability to form a biofilm, lost in the mutant strain, was reconstituted in the complemented strain (Fig. 9). An aggregation assay using settling kinetics of broth cultures, performed as described as previously described (Kjaergaard et al., 2000), has demonstrated that expression AIDA-1 adhesin by wild type and complemented mutant strains of *E.coli* enables auto-aggregation to occur (Fig. 10) (Ravi et al., 2007). (See attached published manuscript.)

Pathogenesis of AIDA-1 *E. coli in vivo*

Experimental infection of neonatal piglets with AIDA-1 E. coli

We conducted a total of 4 experimental infections of colostrum-deprived neonatal piglets with various virotypes of AIDA-1 *E. coli* isolates as listed in Table 5. In the last experiment we included infection with mutant and complemented strains of the mutant AIDA-I negative strain (the AIDA-I gene was deleted from parent wild type to create the mutant strain and than the AIDA-I gene had been reinserted). The mutant and complement strain was developed by our collaborators Drs. Sang-Hyun Kim and Carlton Gyles at the University of Guelph and Drs. Frédéric Berthiaume and Michael Mourez at the University of Montreal.

The complemented strain was evaluated for pathogencity by gastric inoculation of colostrum-deprived newborn pigs kept in a controlled environment, as described previously. Four groups of 6 to 7 piglets each were inoculated with the wild type *E. coli* strain PD20 (AIDA-I$^+$, STb$^+$), the isogenic mutant strain PD20M (AIDA-I$^-$, STb$^+$), the mutant complemented strain PD20C (AIDA-I$^+$, STb$^+$), and a non-pathogenic *E. coli* strain PD71 used as negative control, respectively. Clinical signs of diarrhea were observed and pigs were euthanized at the onset of diarrhea or at 24, 48 and 72 h after infection. At necropsy, gross lesions were noted and intestinal samples were collected for evaluation by histology and immunohistochemistry (jejunum, ileum, cecum and colon) and electron microscopy (ileum and colon). A segment of the ileum was cultured on blood and MacConkey agar plates and the infecting *E. coli* organism confirmed by

immuno-dot blot as previously described. The animals were handled according to the guidelines outlined in *The Guide to the Care and Use of Experimental Animals of the Canadian Council of Animal Care.*

Histology and immunohistochemistry.

The intestinal tissues were fixed in 10% neutral-buffered formalin, processed and embedded in paraffin wax using standard procedures. Four μm sections mounted on glass slides were stained with hematoxylin and eosin (H&E) and Brown and Brenn (B&B) modified Gram stain for histology and bacterial demonstration, respectively. Immunohistochemistry was performed on parallel sections as previously described, using rabbit polyclonal anti AIDA-I (1:2000 dilution) or anti-O143 (1:8000) sera (AIDA-I$^+$ PD20, PD20C and AIDA-I$^-$ strains PD20M are also O143+). Rabbit pre-immune serum was used as the negative control.

Transmission electron microscopy (TEM) and Immuno-gold electron microscopy.

Tissues for TEM were fixed using perfluorocarbon and osmium, and plastic, embedded, as previously described (20.). Ultrathin sections (70-90 nm thickness) of colon from pigs infected with the wild type strain PD20, the mutant PD20M or the complemented strain PD20C were selected, based on immunohistochemical staining firstly of 4 μm paraffin sections to confirm colonization, then of semi-thin sections, and placed on copper grids, as described previously A post-embedding immuno-gold electron microscopy technique modified from Polak and Van Noorden was applied to perflourocarbon fixed, ultrathin sections of colon on nickel grids to visualise expression of AIDA-I protein on the bacterial outer membrane of three *E. coli* strains PD20, PD20C and PD20M. Briefly, the nickel grids were floated for 30 minutes on 4% goat blocker solution (4% Goat serum and 0.1% Tween in PBS), drained and incubated in 1/50 dilution of rabbit anti-AIDA-I IgG and in goat blocker solution for 1hr at room temperature. PD20 infected tissue stained with preimmune rabbit serum was used as the negative control. Grids were then rinsed in 2 changes of 0.1% tween in PBS (PBST), 5 changes of PBS and then in 3 changes of PBS with 0.1% ovalbumin. Subsequently they were incubated for 1 hour in 1/100 dilution of goat anti-rabbit IgG conjugated with 10nm gold particles (British BioCell) in goat blocker solution. The grids then rinsed in 2 changes of PBST, 3 changes of PBS and 4 changes of distilled water. All the rinses were for 5 minutes, under gentle agitation on a magnetic stirrer. Grids were then dried and contrasted with uranyl acetate. All EM was carried out using a Philips 410LS electron microscope.

(Use of the IgG fraction of the rabbit polyclonal anti-AIDA-1 serum, rather than the serum, has been improved quality of results obtained by this technique, and this method has been used for the results depicted in this report.)

Results:

Infection with the AIDA-1 positive, complement *E. coli* strain resulted in pasty diarrhea in neonatal colostrum-deprived pigs comparable to that induced by the wild type AIDA-1 positive strain, the only difference being a slight delay in the time of onset (30 h compared to 20h post-infection, respectively). The results of all the clinical trials are summarized in Table 5

Comparative light and electron microscopic investigation of colonization patterns by the various different virotypes demonstrated biofilm formation with the AIDA-1 positive strains of *E. coli*, i.e the AIDA-1 complement strain as well as the wild type strain, but not the AIDA-1 negative mutant strain. The results indicate that the AIDA-1 adhesin facilitates a distinct pattern of colonization characterized by the formation of a biofilm immediately over the surface of the large

intestinal mucosa (Figure 11 and 12). This biofilm might then interfere with intestinal absorption resulting in diarrhea.

The AIDA-1 adhesin was visualized by immuno-gold electron microscopy and localized to the bacterial surface membrane of both the wild type and the complement AIDA-1 positive strains (Figure 13), confirming morphologically the expression of the AIDA-1 by the complement strain previously demonstrated *in vitro* and consistent with the information in the literature on AIDA-1.

5. *Investigation of AIDA-1 receptor(s) in porcine intestinal mucus*

Based on the intimate interaction between AIDA-I positive *E. coli* and mucus within an intestinal biofilm (Fig. 12), we hypothesized that porcine intestinal mucus contains receptor(s) for AIDA-I adhesin.

Materials and Methods:

AIDA-I-Sepharose matrix preparation and affinity chromatography

AIDA-I affinity column was prepared by coupling of 50 mg of purified AIDA-I adhesin with 3g of freeze-dried CNBr-activated Sepharose 4B powder (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instruction. A negative control column was similarly generated using 3 g of CNBr-activated Sepharose 4B powder without AIDA-I adhesin. Coupling of AIDA-I adhesin to the Sepharose matrix was confirmed by immuno-dot-blotting as previously described (Shahriar et al., 2006a; Shahriar et al., 2006b). The AIDA-I-Sepharose and control Sepharose matrices were packed into separate chromatography columns (XK 16/20, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to be used for isolation of AIDA-I receptors. An ÄKTA purifier 10 chromatography system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was employed with PBS buffer (10mM sodium phosphate, 150mM NaCl, pH 7.2) as the load and wash buffer. Intestinal mucus samples containing 2mg/mL proteins were filtered through 0.45 µm filters (PALL, Gelman Laboratory, MI, USA). Fifteen mL of filtered mucus sample was applied to the AIDA-I-Sepharose column, which was then washed with at least 10 column volumes of PBS buffer until a steady baseline was obtained, as determined by monitoring the OD at 280 nm of the eluate (i.e., <2 mAU). The bound proteins were then eluted from the column with two different elution buffers. The first (low affinity) elution was performed with 3 column volumes of 1M NaCl buffer (in 56mM NaH_2PO_4, 144 mM Na_2HPO_4, pH 7.2) followed by 1 column volume of PBS, while a second (high affinity) elution was done with 3 column volumes of 0.1M Glycine-HCl (pH 2.5). These two elution fractions were collected and pooled separately, dialyzed for 48 h at 4°C against deionized H_2O, using 1 kDa cut-off cellulose dialysis membranes (Spectra/Por, Spectrum Laboratories Inc, CA, USA), and then lyophilized (Labconco Corp, MO, USA). The same procedure was repeated for the negative control Sepharose column. The entire purification procedure was performed at 4°C.

ELISA assay

ELISA assay was used to determine interaction between isolated AIDA-I receptors and purified AIDA-I adhesin. The protein concentrations of the isolated AIDA-I receptors, crude mucus, and bovine albumin (negative control) were adjusted to 0.11mg/ml. A series of dilutions were prepared for albumin, mucus, and AIDA-I receptors eluted by 1M NaCl from AIDA-I-Sepharose affinity column as described above. 96-well plates were coated with 100 µL of the above-prepared dilutions (2 wells for each dilution), incubated at room temperature for 2 h, washed 3 times with PBST (PBS with 0.05% Tween-20), blocked with PBS-10% fetal calf serum (pH 7.2) for 2 h at room temperature, and washed 3 times with PBST. Fifty µL of purified

AIDA-I adhesin (12μg/ml) was added to each well and incubated for 2 h at room temperature. Unbound AIDA-I adhesin was removed from the wells by four consequential washes. Bound AIDA-I adhesin was detected in each well by sequential incubation with primary rabbit polyclonal anti-AIDA-I immunoglobulins (1:4000), secondary goat anti-rabbit IgG conjugated HRP (1:2000, Bio-Rad Laboratories, Ltd. Mississauga, ON), and TMB Peroxidase EIA substrate (Bio-Rad Laboratories, Ltd. Mississauga, ON), and subsequently quantified by a microplate reader (MPM III, Bio-Rad Laboratories, Ltd. Mississauga, ON) at 450 nm. The data were collected, analyzed and plotted.

Overlay Westernblot Overlay

Western blot was used to confirm interaction between the isolated AIDA-I receptors and purified AIDA-I adhesin or AIDA-I positive *E. coli*. The isolated AIDA-I receptors were separated by one- and two-dimensional SDS-PAGE and electroblotted onto PVDF membranes using small (for 1-D PAGE) or large (for 2-D PAGE) electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. The PVDF membrane with the blotted sample was subjected sequentially to the following procedures: blocking of any remaining protein binding sites with blocking buffer (2% non-fat dry milk, 0.1% Tween-20 in PBS); incubation with purified AIDA-I adhesin (2.5 μg/ml) or AIDA-I positive *E. coli* ($1x10^5$ CFU/ml; CFU/ml=$OD_{620}x2.5x10^8$) in blocking buffer for 90 minutes at room temperature with gentle shaking; five 15-min washes with PBS Tween-20 (PBST) buffer (0.1% Tween-20 in PBS); incubation with purified primary rabbit polyclonal anti-AIDA-I antibodies (1:5000) for 90 minutes; five 15-min washes with PBST; incubation with secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (1:20,000, Sigma Chemical Co, St Louis, MO, USA) for 90 minutes; three 15-min washes with PBST; and exposure to a substrate solution (Immun-Star Substrate, Bio-Rad Laboratories, Mississauga, ON). Chemiluminescent signal demonstrating AIDA-I receptors was detected by exposure of the PVDF membrane to an X-ray film (Kodak X-Omat AR Film, Eastman Kodak Company, Rochester, NY, USA).

Results

AIDA-I adhesin isolated by heat extraction and purified by gel filtration was coupled to activated sepharose and used for affinity isolation of AIDA-I binding proteins from intestinal mucus. Several mucus proteins were eluted from the AIDA-I-Sepharose matrix by a low affinity elution (1M NaCl) (Fig. 14, Lane 1), and subsequently 65 and 120 kDa AIDA-I-binding proteins (p65 and p 120) were eluted by high affinity elution (low pH buffer) (Fig. 14, Lane 2). Several proteins ranging form 30 to 60 kDa and from 14 to 25 kDa were eluted from both the AIDA-I-Sepharose and the Sepharose only (negative control) matrices (Fig. 14, Lane 1, 2, and 3), accordingly, they were considered to be Sepharose-binding proteins, but not AIDA-I binding proteins.

More intense binding of purified AIDA-I adhesin to the protein fraction eluted by 1M NaCl from AIDA-I-Sepharose matrix than to crude mucus confirmed that affinity chromatography employed for isolation of AIDA-I receptors was effective (Fig. 15); and similarly, the more intense binding to the protein fraction eluted by 1M NaCl from AIDA-I-Sepharose matrix exhibited by AIDA-I positive *E. coli* than by either AIDA-I negative mutant *E. coli* or F4 positive *E. coli* confirmed that the isolated fraction contained proteins interacting specifically with AIDA-I adhesin (Fig. 16). The protein fractions isolated by low and high affinity elution from AIDA-I-Sepharose matrix contained several proteins; therefore, Overlay Western Blotting was used to demonstrate that purified AIDA-I adhesin or AIDA-I positive *E.*

coli bound exclusively to p65 and p120 separated by 1D or 2D SDS-PAGE and blotted onto PVDF membrane (Fig. 17 and 18). Based on 2D SDS-PAGE page migration, p120 has 3 isoforms with isoelectric points (pI) ranging from 4.8 to 5.2, and p65 has several isoforms, pI of which ranges from 5.2 to 5.7.

p65 and p120 were excised from 1D SDS-PAGE gels, submitted for LCMS/MS, and peptide fragment mass fingerprints of p120 were matched with Mascot database search to DNA damage binding protein 1 (gi|418316), splicing factor 3b (gi|19527174), and IgG Fc binding protein (gi|4503681) (Table 6), whereas peptide fragment mass fingerprints of p65 were matched predominantly to various cytokeratins of stratified squamous epithelial cells (commonly detected as a contaminant protein by LCMS/MS protein identification procedure) and occasionally to cytokeratins of simple epithelia (i.e. cytokeratins 8 and 19) (Table 4.1).

Conclusion:

We have demonstrated that porcine intestinal mucus contains 65 and 120 kDa proteins (p65 and p120) that bind with high affinity to AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* isolates and that AIDA-I positive *E. coli* binds to these proteins with higher affinity than do an AIDA-I negative mutant and F4 positive *E. coli*. Based on LCMS/MS data, the identity of p65 was not determined, whereas p120 was matched to two nuclear proteins (namely, DNA damage binding protein and splicing factor 3b) and one cytoplasmic protein, which is an IgG Fc binding protein. Based on similar amino acid homology, molecular weight, structural similarity to mucin and evidence of being secreted by goblet cells into the intestinal lumen, we think that this IgG Fc binding protein is most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin. Further studies *in vivo* are needed to determine the role of IgG Fc binding protein in intestinal colonization and pathogenesis of diarrhea induced by AIDA-I positive *E. coli*.

6. *Age-associated expression of intestinal receptors for AIDA-1 E. coli.*

Due to unsuccessful development of polyclonal antibodies against the above-described p65 and p120 AIDA-I receptors, we failed to determine the age-associated expression of intestinal receptors for AIDA-1 *E. coli*. As we originally proposed, rabbits were immunized with p65 and p120 according to previously reported procedure used successfully many times in our laboratory for various other proteins including AIDA-I adhesin (Fang et al., 2005; Shahriar et al., 2006a; Shahriar et al., 2006b). Nevertheless, antibody titers developed against these proteins by two different rabbits were inadequate for any immunodetection technique even after multiple boost injections that used up the entire sample with isolated AIDA-I receptors. It is possible that immunogenicity of these proteins is very low, therefore, together with commercial TiterMax Gold adjuvant they did not initiate appropriate immunological response in rabbits. In retrospect, we should have tried to use Freund's adjuvant, even though their use is discouraged by both the University of Saskatchewan Animal Care Committee and Canadian Council on Animal Care. Despite difficulties encountered in this part of our investigation, we hope to continue with this project. We are in the process of requesting antibodies against human IgG Fc binding protein reported by Harada et al. If there is a cross reactivity between human and porcine IgG Fc binding proteins, that we suspect serve as AIDA-I receptor in porcine intestinal mucus based on the above-indicated study, we will be able to determine the age-associated expression of IgG Fc binding protein by porcine intestinal mucosa. In addition to that, we will be able to employ competitive inhibition binding assays in vitro to determine further if IgG Fc binding protein is genuine AIDA-I receptor.

7. *Prevalence of AIDA-1 E. coli in modern swine industry*

To investigate prevalence of AIDA-1 *E. coli* infection in modern swine industry, we have established collaboration with Gallant Custom Laboratories Inc., Cambridge, Ontario, that provides microbiological services to swine practitioners in Alberta, Saskatchewan and Manitoba. Our team met with the major swine practitioners in the Prairie Provinces at the annual meeting of Western Canadian Association of Swine Practitioners in October, 2004, and discussed/explored the best approach to determine clinical significance and prevalence of AIDA-I *E. coli* infection. A total of 110 *E. coli,* isolated from problematic cases of F4-negative *E. coli* diarrhea in pigs which tested negative for F4 fimbria by standard slide agglutination test, were used in this study. These cases originated from commercial swine herds located in the Western Canadian prairie provinces: Saskatchewan, Manitoba and Alberta, and were submitted to Gallant Custom Laboratories, Ontario, and Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan between 1999-2005. The clinical data for these isolates were obtained by contacting the practitioners and asking them to fill out a questionnaire regarding clinical presentation and all 110 *E. coli* isolates were tested by M-PCR for various known virulence factors of *E. coli*. Nine (8.2%) isolates of 110 F4 negative *E. coli* associated with clinical diarrhea were positive for AIDA-I adhesin. The prevalence of other fimbrial adhesins in the decreasing order include: 1.8% each for F41, F18 and F5, and 0.9% for F6. The prevalence of other non-fimbrial adhesins in the present study include: 8.2% AIDA-I and 3.6% for EAE. 22.7% of isolates carried at least one toxin and 13.6% carried at least one of the adhesins and toxins. 61.8% of the *E. coli* were negative for all tested common adhesin and toxin genes (Table 7). Clinical information was available for 35 isolates (5 AIDA-I$^+$ and 30 AIDA-I$^-$) representing 18 diarrhea cases. At the farm level, the clinical problem of diarrhea among these 18 cases was persistent (50%), recurrent (22.2%), at single point of time (16.6%) or sporadic (11.1%). These cases represented three different age (in days) groups: 0-7 d (n=9), 8-14 d (n=4) and 21-60 d (n=5), in farrow-finisher swine operations. There were 5 AIDA-I$^+$ *E. coli* isolates representing 3 (16.7%) clinical cases. All the AIDA-I$^+$ *E. coli* belonged to the 21-60 days old group while 87% of the AIDA-I$^-$ *E. coli* were from the pigs younger than 14 days old.

8. *Summary*

AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* clinical isolates is an acidic 100 kDa protein consisting of five isoforms, which has relatively high amino acid homology with AIDA-I adhesin isolated from human AIDA-I positive *E. coli* isolates (Fang et al., 2005). Using various AIDA-I *E. coli* strains (wild positive, mutant negative, complemented positive) we demonstrated that AIDA-I adhesin is necessary for diffuse adherence to HeLa cells, enhancement of bacterial autoaggregation and biofilm formation *in vitro*. In addition, we demonstrated *in vivo* that AIDA-I adhesin is a significant virulence factor necessary for intestinal colonization, induction of biofilm formation and development of diarrhea (Ravi et al., 2007). Based on intimate interaction between AIDA-I positive *E. coli* and mucus we hypothesized and subsequently demonstrated that porcine intestinal mucus contains AIDA-I receptors (p65 and p120). The identity of p65 was not determined based on LCMS/MS data, whereas p120 was matched to IgG Fc binding protein which is hypothesized to serve as AIDA-I receptor in porcine mucus (Fang et al., 2007). Based on our *in vivo* experiments and based on survey results, we suspect that AIDA-1 positive *E. coli* infection may interfere with optimal growth of neonatal and postweaning piglets and cause economical loses in modern swine industry primarily due to diarrhea. However, mortality does not seem to be a clinical feature in uncomplicated neonatal infections by

AIDA-I positive *E. coli*. Based on limited survey data, it seems that AIDA-I positive E. coli is more common in weaned piglets. If we obtain anti IgG Fc binding protein antibody from Harada et al we will be able to determine if AIDA-I receptors are more abundant in weaned than in suckling piglets. We will also be able to demonstrate co-localization of AIDA-I adhesin and IgG Fc binding protein *in vivo* using immuno-gold electron microscopy. In addition, it would be beneficial to characterize experimental challenge of weaned piglets with AIDA-I *E. coli*, since in this study we used only neonatal piglets. Our future studies will also determine if colostral anti-AIDA-I antibody can prevent diarrhea induced AIDA-I *E. coli* in neonatal piglets and if vaccination using AIDA-I antigens can prevent disease in weaned pigs.

EXPENSE STATEMENT

The official Statement of Expenditures prepared by the Financial Services, University of Saskatchewan will be mailed directly to you.

DISSEMINATION / COMMUNICATION OF RESULTS

Papers

1. Y. Fang, M. Ngeleka, D.M. Middleton, E. Simko, 2005. Characterization and immuno-detection of AIDA-1 adhesin isolated from porcine *Escherichia coli.* Veterinary Microbiology, 109, 65-73. (See attached reprint)
2. M. Ravi, M. Ngeleka, S-H. Kim, C.L. Gyles, F. Berthiaume, M. Mourez, D.M. Middleton, E. Simko. 2007. Contribution of AIDA-I to the pathogenicity of a porcine diarrheagenic *Escherichia coli* and to intestinal colonization through biofilm formation in pigs, Veterinary Microbiology, 120, 308-319
3. Y. Fang, M. Ngeleka, D.M. Middleton, E. Simko. Isolation and identification of AIDA-I receptors in porcine intestinal mucus. Veterinary Microbiology – Accepted with minor revisions, March 2007.
4. M. Ravi, M. Ngeleka, D.M. Middleton, E. Simko. Prevalence and clinical significance of AIDA-I associated virotypes among non-F4 *E. coli* isolates from problematic cases of diarrhea in pigs – manuscript in preparation (attached first draft)

Thesis:

5. M. Ravi, MSc Thesis: Pathogenesis and clinical significance of AIDA-I *E. coli* in diarrhea of pigs, University of Saskatchewan, 2006
6. Y. Fang, MSc Thesis: Characterization of porcine AIDA-I adhesin and its receptors, University of Saskatchewan, to be defended in April 2007.

Prsentations, Abstract and Posters

7. M. Ravi, M. Ngeleka, S-H. Kim, C.L. Gyles, F. Berthiaume, M. Mourez, D.M. Middleton, E. Simko. Role of AIDA-I adhesin in pathogenesis of E. coli induced diarrhea in pigs.
   - 57th Annual Meeting of the American College of Veterinary Pathologists, Tucson, Arizona 2006 – Poster and Abstract - 2nd Prize Young Investigator Award for Experimental Diseases
   - 2005 Western Canadian Association of Swine Practitioners – Paper and Presentation

8. Y. Fang, M. Ngeleka, D.M. Middleton, E. Simko. Characterization of AIDA-I adhesin and its receptors and immuno-detection of AIDA-I positive *Escherichia coli.* 5th Annual Meeting of the Canadian Animal Health Laboratorians Network, Ottawa, Ontario 2006 - Abstract and Presentation – Awarded for the Best Graduate Student Presentation
9. M. Ravi, M. Ngeleka, R. Mainar-Jaime, S-H Kim, C. Gyles, F. Berthiaume, M. Mourez, D.M. Middleton and E. Simko. Role of AIDA-I *E. coli* in the pathogenesis of diarrhea: prevalence of AIDA-I associated virotype among non-F4 (K88) *E.coli* isolates from cases of problematic diarrhea in pigs. - 5th Annual Meeting of the Canadian Animal Health Laboratorians Network, 2006 - Abstract and Presentation
10. M. Ngeleka, M. Ravi, E. Simko, D.M. Middleton, 2005. Diagnosis of neonatal diarrhea due to *E. coli* in piglets and calves: A western Canadian experience. Canadian Animal Health Laboratory Network, St-Hyacinthe, Quebec – Presentation.
11. Y. Fang, F. Shahriar, M. Ngeleka, D.M. Middleton and E. Simko, (March 14, 2003). Isolation of AIDA-I adhesin and detection of AIDA-I positive *Escherichia coli* isolates from pigs. WCVM Graduate Student Poster Day, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada – Poster.
12. WCVM Annual Research Report 2003, Research in Veterinary Pathology focused on neonatal porcine diarrhea.

ACKNOWLEDGEMENT

Financial support provided by the Saskatchewan Agriculture Development Fund was appropriately acknowledged and promoted in every instance that this research was published, presented or discussed (see attached reprints and copy of a few posters).

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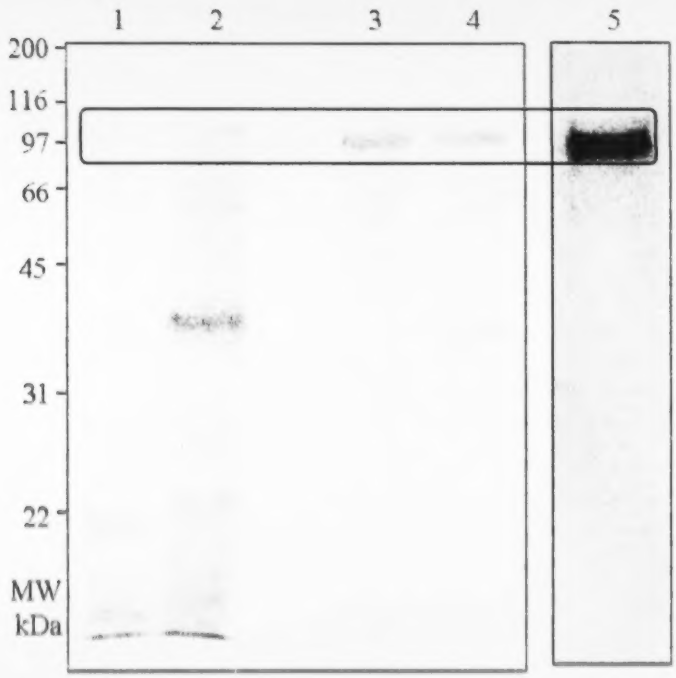


Figure 1: Coomassie-stained SDS PAGE and Western blot of AIDA-I proteins. Surface bacterial proteins harvested from porcine AIDA-I *E. coli* isolates PD58 and PD20 (Lane 1 and 2, respectively); Semi-purified AIDA-I proteins from PD58 and PD20 (Lane 3 and 4); AIDA-I protein isolated from PD58 and detected by Western blot using rabbit polyclonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I *E. coli* (Lane 5). AIDA-I protein bands are encircled.

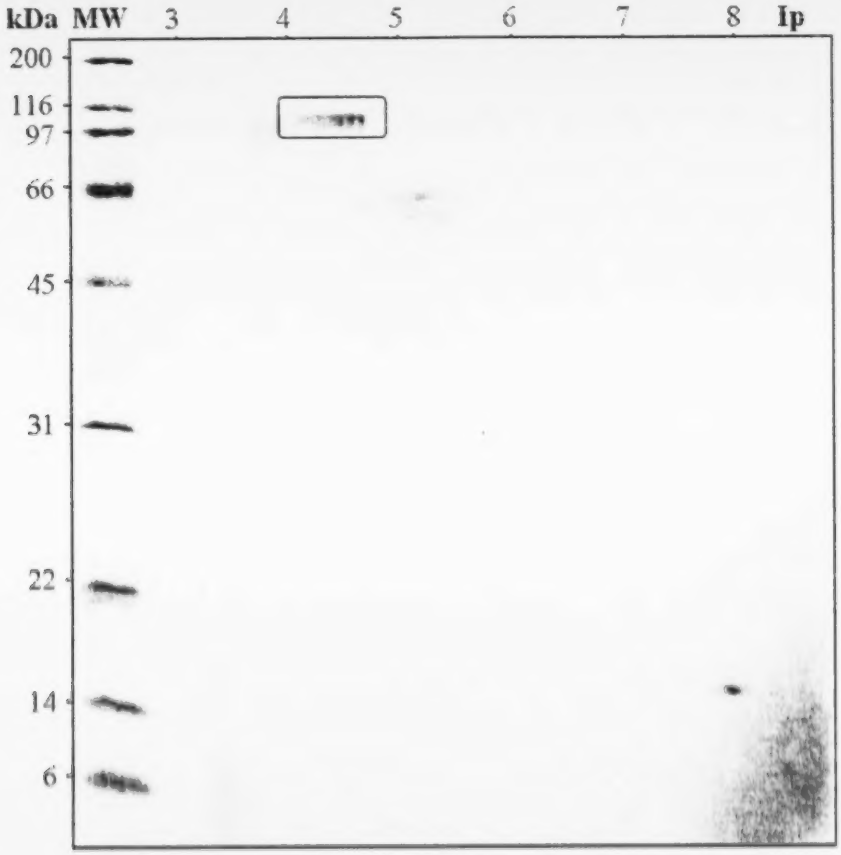


Figure 2: Coomassie-stained AIDA-1 protein isolated from porcine AIDA-1 *E. coli* and separated on two dimensional SDS PAGE. Isoforms of AIDA-1 protein are encircled

PD20 AIDA-1 *E. coli*

AIDA-1 *E. coli* strain 2787

Figure 3: Immuno-dot-blot detection of porcine AIDA-1 *E. coli* isolate PD20 and failure of detection of human AIDA-1 *E. coli* strain 2787 using rabbit polyclonal antiserum raised against AIDA-1 protein isolated from porcine AIDA-1 *E. coli*.

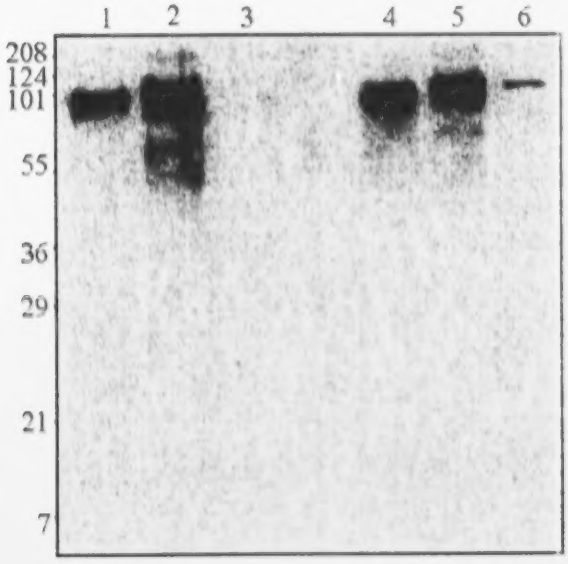


Figure 4: Western blot detection of AIDA-1 protein from porcine isolate PD20 and human AIDA-1 *E. coli* using rabbit polyclonal antiserum raised against AIDA-1 protein isolated from porcine AIDA-1 *E. coli*. Proteins in Lane 1-3 were separated by reducing SDS PAGE and proteins in Lane 4-6 were separated by non-reducing PAGE. Isolated AIDA-1 protein from porcine AIDA-1 *E. coli* isolate PD20 (Lanes 1 and 4); Surface proteins harvested from porcine AIDA-1 *E. coli* isolate PD20 (Lanes 2 and 5); Surface proteins harvested from human AIDA-1 *E. coli* strain 2787 (Lanes 3 and 6).

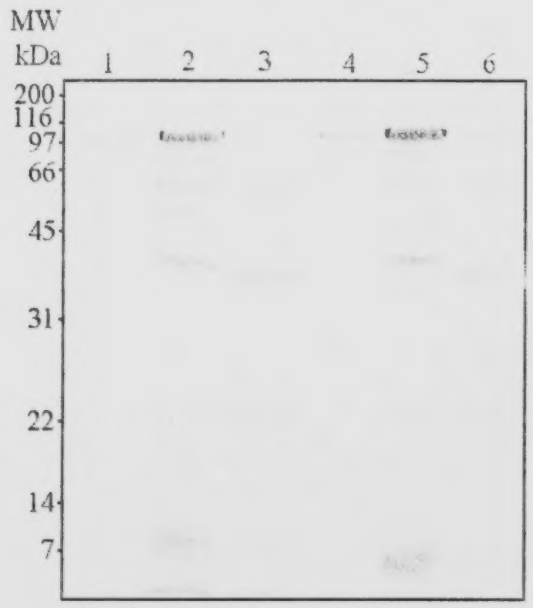


Figure 5: AIDA-1 protein from porcine isolate PD20 and human AIDA-1 *E. coli* strain 2787 separated by non-reducing (Lane 1-3) and reducing (Lane 4-6) SDS PAGE and visualized by Coomassie Brilliant Blue staining. Isolated AIDA-1 protein from PD20 porcine AIDA-1 *E. coli* isolate PD20 (Lanes 1 and 4); Surface proteins harvested from porcine AIDA-1 *E. coli* isolate PD20 (Lanes 2 and 5); Surface proteins harvested from human AIDA-1 *E. coli* strain 2787 (Lane 3 and 6).

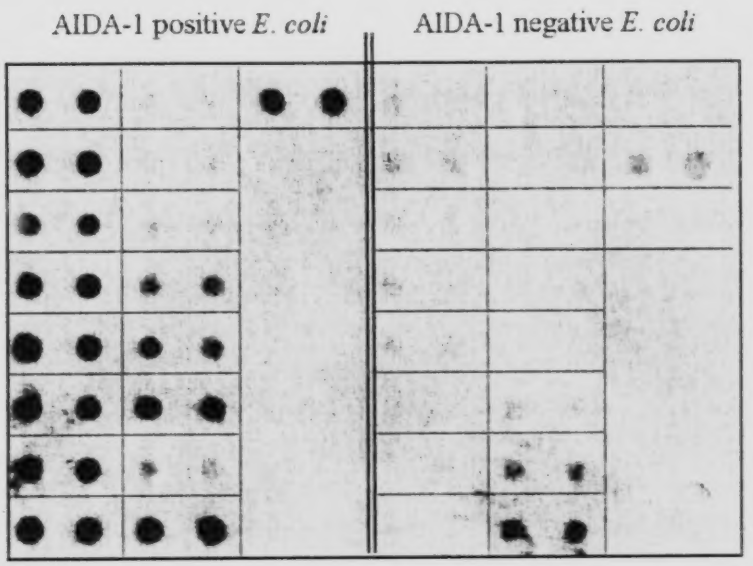


Figure 6: Immuno-dot-blot detection of AIDA-1 protein on bacterial surfaces of porcine *E. coli* isolates determined to be positive or negative for AIDA-1 gene by PCR. *E. coli* isolates were dot-blotted in duplicates.

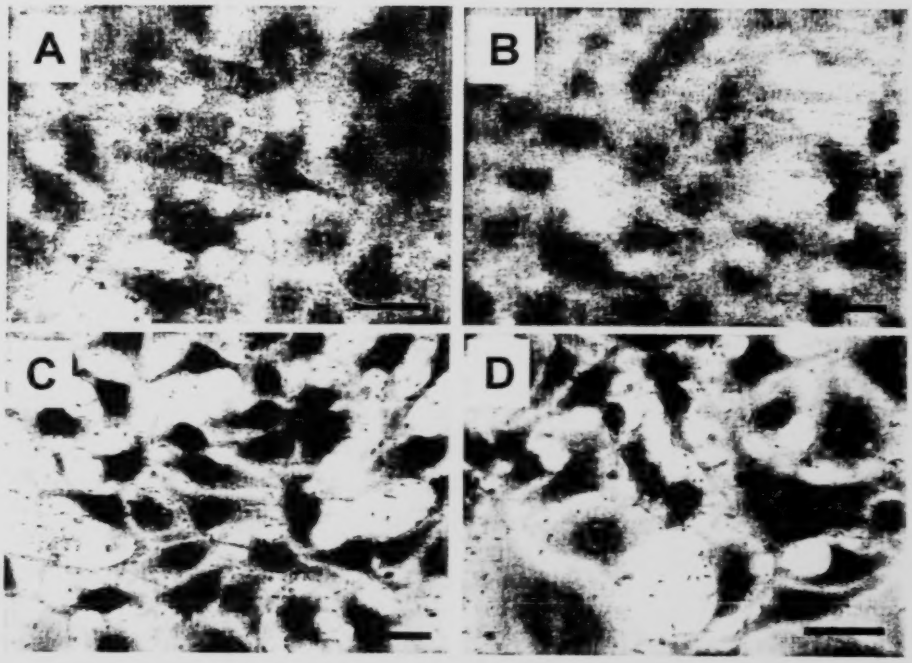


Figure 7. AIDA-I mediated attachment to HeLa cells is demonstrated by diffuse adherence by the AIDA-I+ wild (A) and complemented (B) strains. In contrast, minimal adherence to HeLa cells occurred with AIDA-I- mutant (C) and negative control (D) strains. Bars = 25 μm.

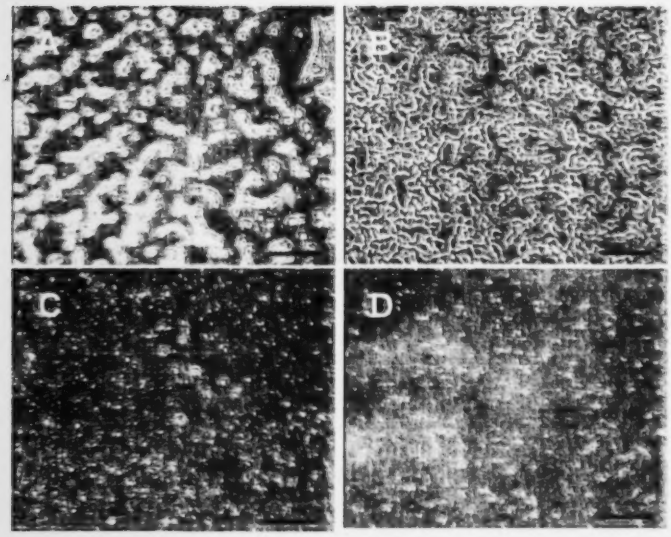


Figure 8. Phase contrast microscopy of *E.coli* strains grown in TSB on polystyrene dishes showing bacterial aggregation and biofilm formation by the AIDA-I$^+$ strains, wild type strain PD20 (A) and the complemented mutant PD20C (B). In contrast, neither bacterial aggregation nor biofilm formation was observed with the AIDA-I$^-$ mutant PD20M (C) or the negative control PD71 strains (D). Bars = 50 □m.

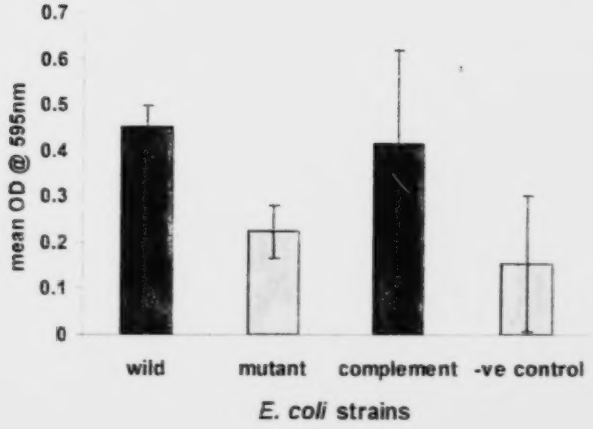
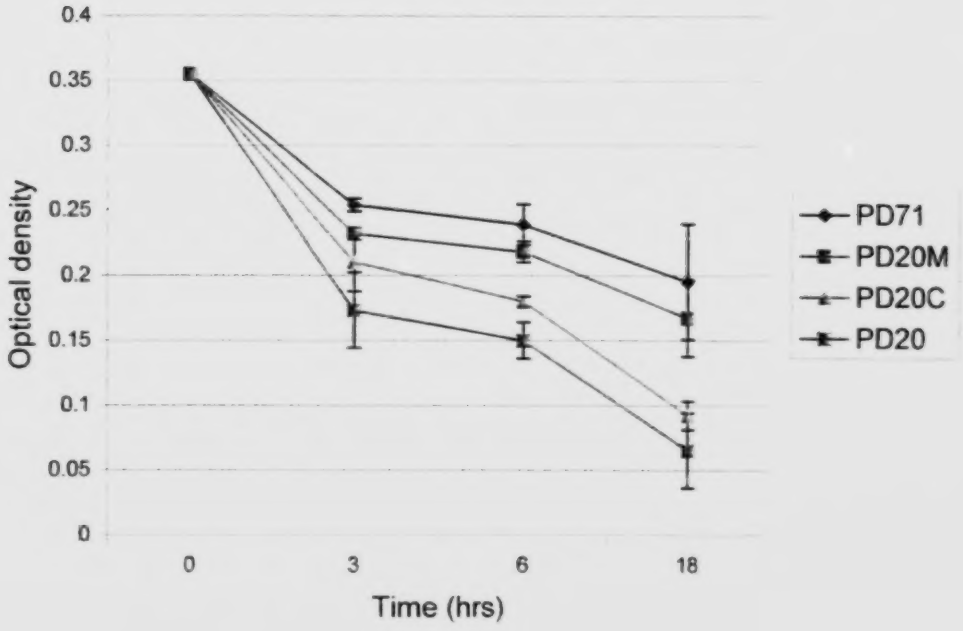


Figure 9. Biofilm formation was significantly greater ($p<0.5$) for both wild and complemented AIDA-I$^+$ strains than for AIDA-I$^-$ mutant and negative control strains. Data represent mean optical density (OD) with standard deviations from 3 independent experiments.



Figigure 10 The auto-aggregation assay performed in TSB medium, was assessed by the settling kinetics of bacteria and evaluated at an optical density of 600 nm The AIDA-I⁺ wild type strain PD20 and the complemented strain PD20C settle faster than the AIDA-I⁻ mutant PD20M or the negative control stain PD71. Results indicate the mean ± standard error of the data obtained from two independent experiments.

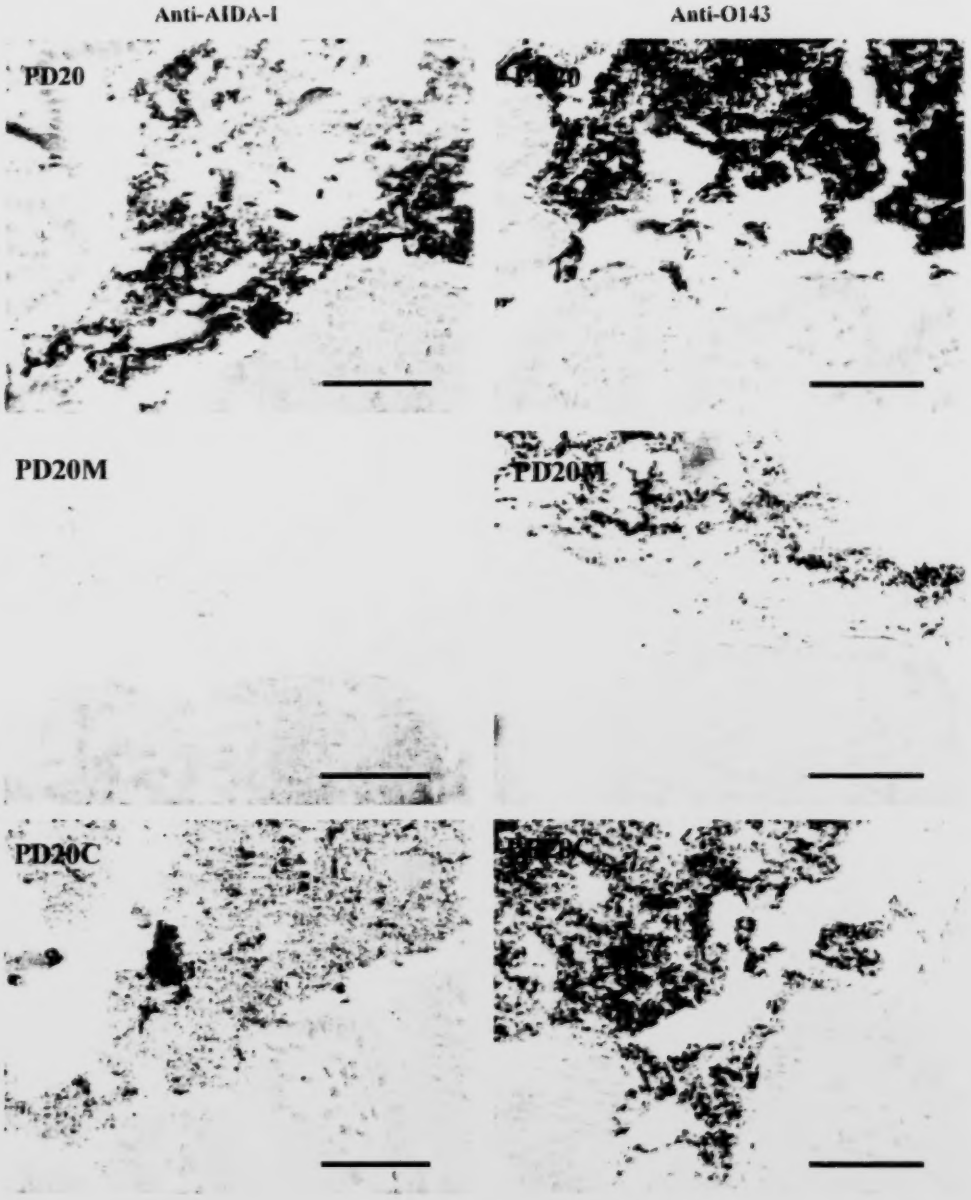


Figure 11. Sections of colon shows heavy bacterial colonization and bacteria intimately associated with the mucosa of pigs infected with the wild type strain PD20 and the complemented strain PD20C using AIDA-I antiserum (1:500) and O143 antiserum (1:4000) respectively). In contrast, specific staining is absent and markedly lesser colonization is seen in the colon of pigs infected with the AIDA-I- mutant PD20M when stained with AIDA-I or with O143 antiserum respectively. Bars = 50 mm.

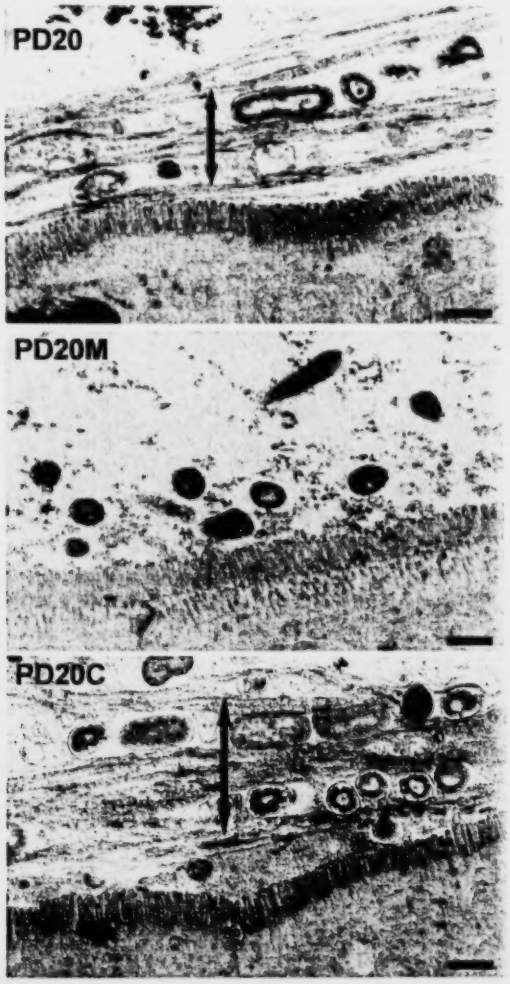


Figure 12 Transmission electron micrographs of perfluorocarbon fixed, epon-embedded colonic epithelium show bacteria stratified within mucus layers, forming a biofilm (arrows) in pigs infected with AIDA-I+ wild type PD20 or the complemented strain PD20C. In contrast, with the AIDA-I- mutant strain PD20M, no biofilm was formed and bacteria were irregularly distributed above the colonic enterocytes. Bars = 1 mm.

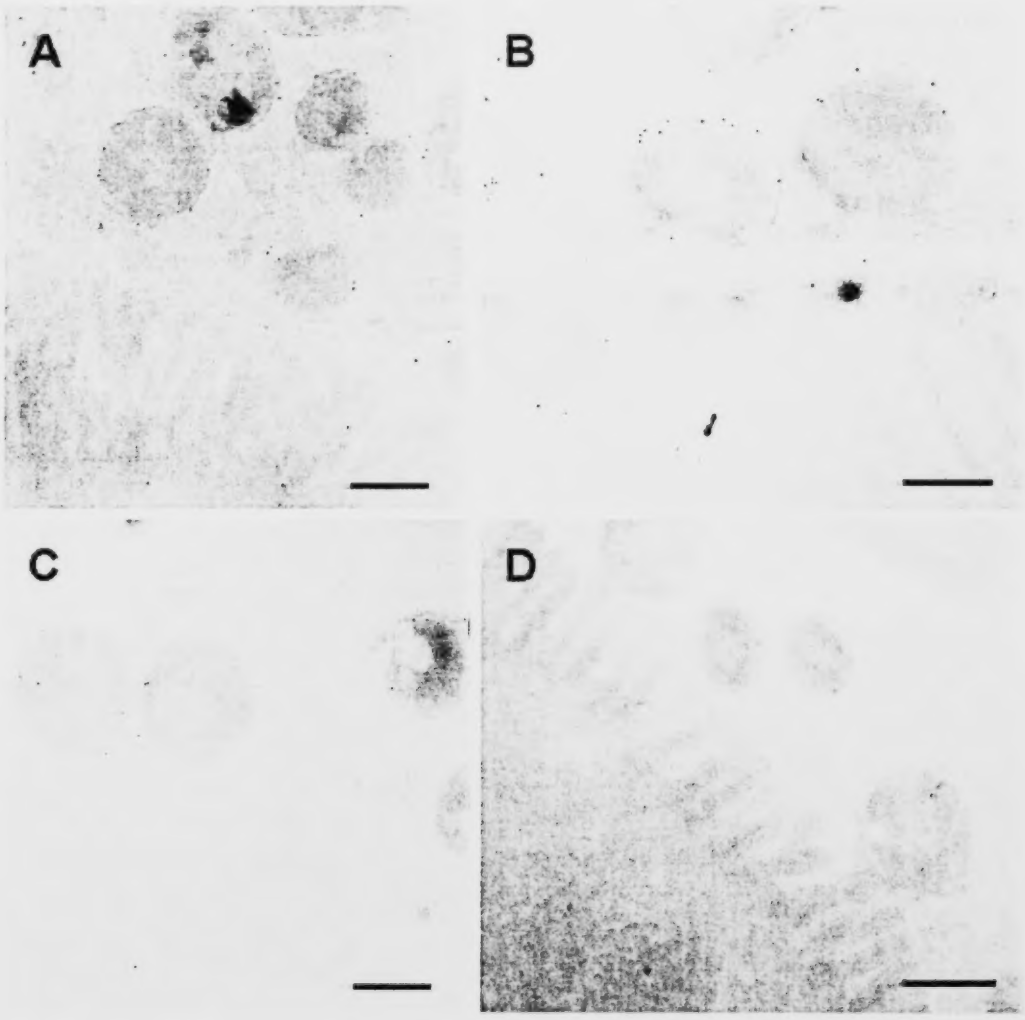


Figure 13. Localization of the AIDA-I adhesin observed by TEM immunogold labeling of perfluorocarbon fixed and epon embedded colonic epithelium using anti-AIDA-I IgG antibody showing gold particles (10nm) on the outer membrane surface the wild type strain PD20 (A), the complemented strain PD20C (B) and the mutant strain PD20M (C). Rabbit pre-immune serum used as a negative control to stain PD20 infected tissue (D), bars = 0.5 μm.

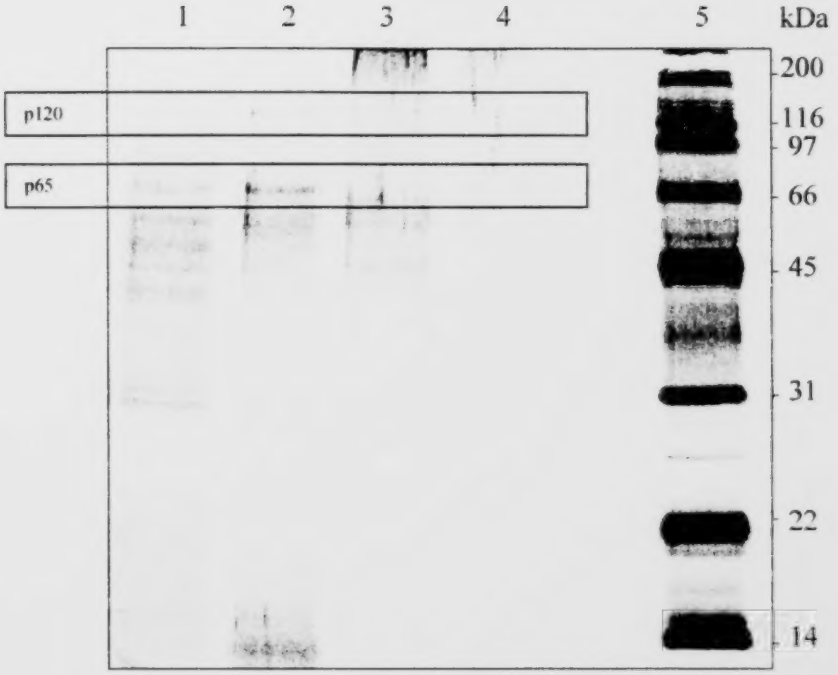


Figure 14. Silver stained reduced SDS-PAGE of AIDA-I binding proteins eluted from AIDA-I-Sepharose matrix with 1M NaCl (Lane 1) followed by pH 2.5 elution buffer (Lane 2), and Sepharose binding proteins eluted from negative control affinity matrix (Sepharose only) with 1M NaCl (Lane 3) followed by pH 2.5 elution buffer (Lane 4). Molecular weight markers (Lane 5).

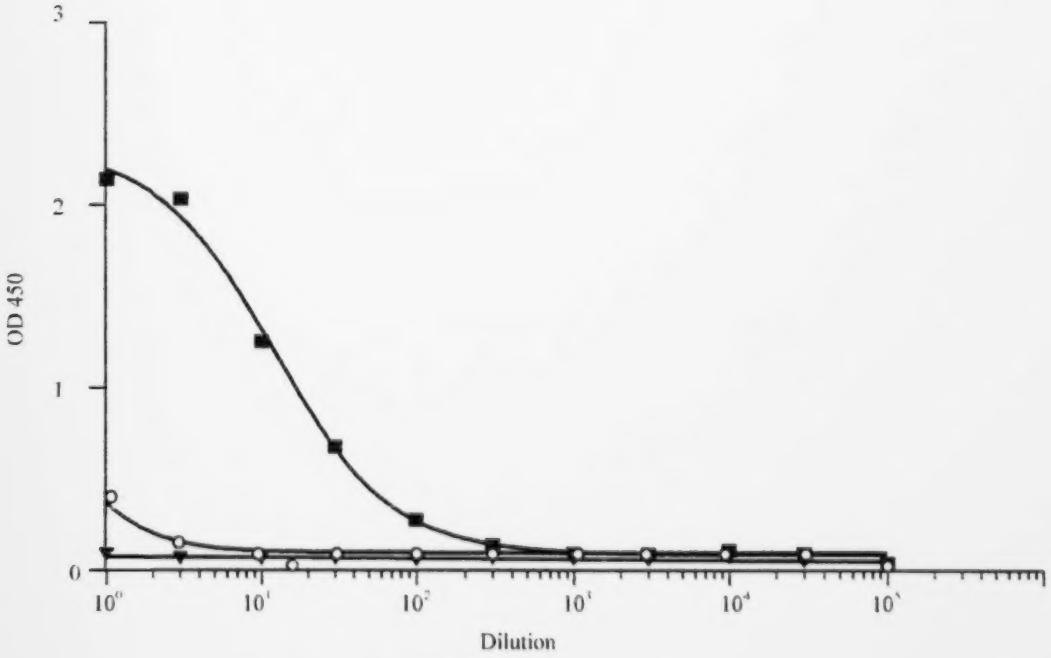


Figure 15. Interaction between purified AIDA-I adhesin and 1) AIDA-I binding proteins eluted by 1M NaCl (■), 2) crude porcine intestinal mucus (○), and 3) albumin (negative control) (▼). AIDA-I adhesin interacted most intensely with AIDA-I binding proteins isolated by affinity chromatography. Quantification of interaction was determined by ELISA assay using polyclonal anti-AIDA-I antibody.

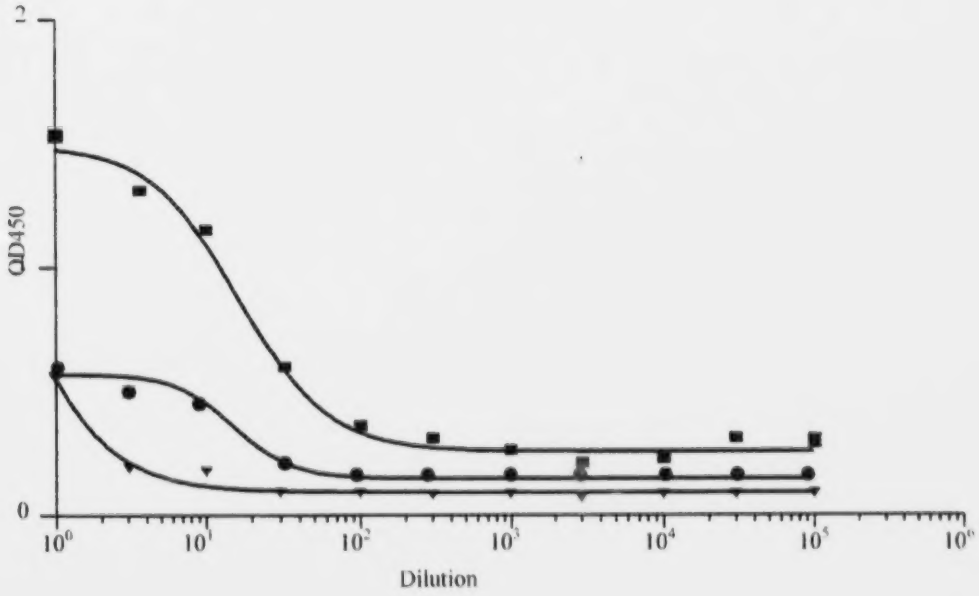


Figure 16. Interaction between AIDA-I binding proteins eluted by 1M NaCl and 1) AIDA-I positive *E. coli* (■), 2) AIDA-I negative mutant *E. coli* (●), F4 positive *E. coli* (▼). AIDA-I binding proteins interacted most intensely with AIDA-I positive *E. coli*. Quantification of interaction was determined by ELISA assay using polyclonal anti-anti-F4 serum for *E. coli* $F4^+$ and AIDA-I antibody polyclonal serum raised against whole AIDA-1 positive *E. coli* for detection of both AIDA-1 positive and AIDA-I negative mutant *E. coli*.

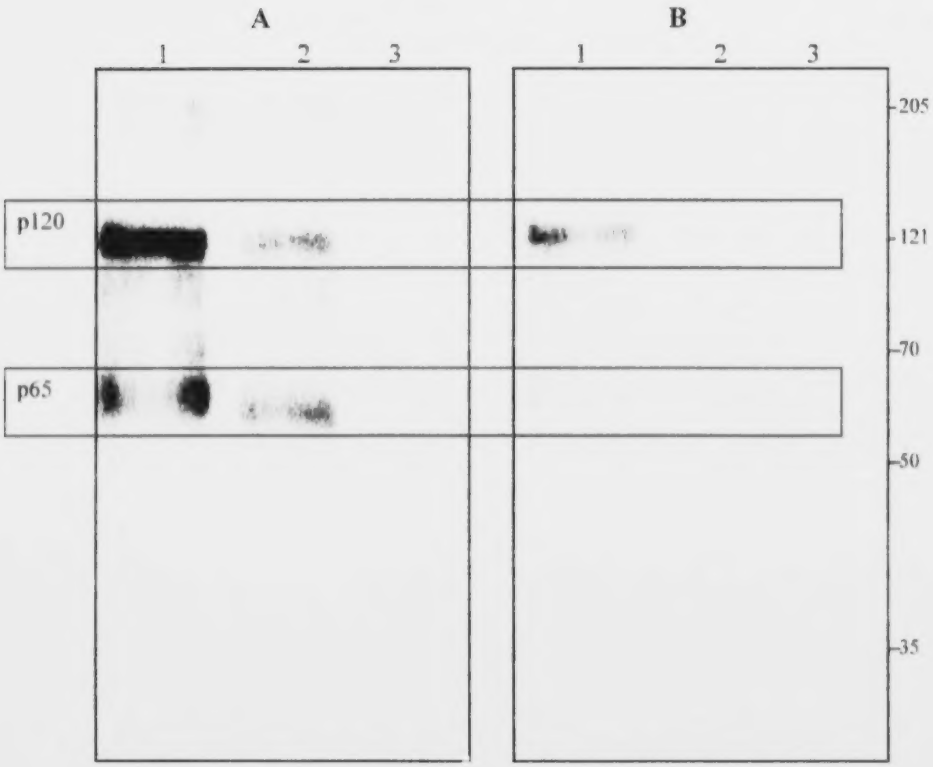


Figure 17. Overlay Western Blot detection of p65 and p120 AIDA-I binding proteins. AIDA-I binding proteins eluted by 1M NaCl (Lane 1A and 1B) and low pH elution buffer (Lane 2A and 2B), and crude intestinal mucus (Lane 3A and 3B) were separated by reduced one-dimensional SDS-PAGE and electroblotted to PVDF membrane. P65 and p120 were detected by purified AIDA-I adhesin (A) and AIDA-I positive *E. coli* (B) as described in Material and Methods.

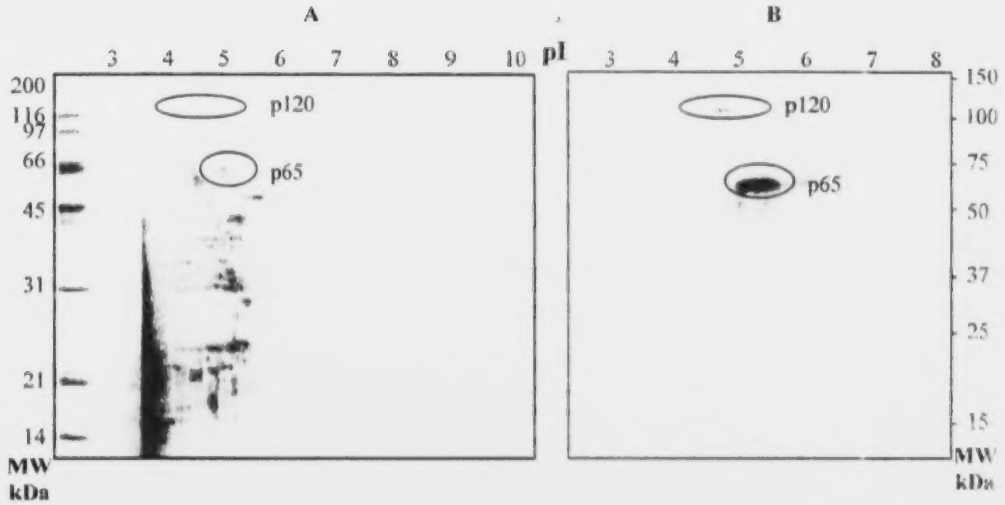


Figure 18. Silver-stained two-dimensional SDS-PAGE of AIDA-I binding proteins eluted by 1M NaCl (A) and Overlay Western Blot detection of p65 and p120 AIDA-I binding proteins using purified AIDA-I adhesin (B) as described in Material and Method.

Table 1 Comparison of amino acid sequences determined by LCMS/MS for fragments of AIDA-1 protein isolated from the porcine AIDA-1 *E. coli* isolate PD58 with AIDA-1 protein from the human AIDA-1 *E. coli* strain 2787 (Benz and Schmidt, 1992a). Shaded pairs are different. Identity = 39/45 (86.6%)

		338										352						473							480	
Human	...	A	I	N	T	L	V	S	D	G	G	Y	Q	H	I	R	...	A	E	V	Y	S	G	G	K	...
PD 58	...	A	L	N	T	L	V	S	D	G	G	S	Q	H	L	R	...	A	E	V	Y	S	G	G	K	...

		679						685			822														836	
Human	...	N	S	A	G	R	L	R	...		B	L	L	S	A	T	V	N	G	S	L	V	N	N	K	...
PD 58	...	N	S	A	G	M	L	R	...		K	L	L	S	A	T	V	D	G	S	L	V	N	N	K	...

Table 2 Comparison of amino acid sequences determined by LCMS/MS for fragments of AIDA-1 protein isolated from the porcine AIDA-1 *E. coli* isolate PD20 with AIDA-1 protein from the human AIDA-1 *E. coli* strain 2787 (Benz and Schmidt, 1992a). Shaded pairs are different. Identity = 76/97 (78.4%)

	112					117			340										352		388				393					
Human	..	G	A	T	I	S	T	...	N	T	L	V	S	D	G	G	Y	Q	H	I	R	...	R	V	L	S	D	G	...
PD 20	..	G	A	T	V	S	T	...	N	T	L	V	S	D	G	G	S	Q	H	L	R	...	B	V	L	S	D	G	...

	396										405		408						414		520									530	
Human	..	R	G	T	I	L	N	N	S	G	R	..	V	S	N	G	G	V	S	..	F	A	G	N	V	V	G	T	I	L	N	..
PD 20	..	B	E	T	L	L	N	N	S	G	R	..	V	S	D	G	G	V	S	..	F	A	G	N	V	V	G	T	L	L	N	..

	691							698		797						804		825												836	
Human	..	T	I	N	G	T	T	T	I	..	G	S	I	V	I	N	N	S	..	S	A	T	V	N	G	S	L	V	N	N	K	..
PD 20	..	T	V	D	G	S	T	T	L	..	G	S	L	V	S	D	N	S	..	S	A	T	V	D	G	S	L	V	N	N	K	..

	847								855			1177				1183															
Human	..	A	G	N	T	L	T	V	S	N	...		T	V	V	Q	G	A	G	..											
PD 20	..	A	G	N	V	V	T	V	V	N	...		T	V	V	Q	A	A	G	..											

Table 3 Comparison of amino acid sequences determined by LCMS/MS for a single fragment representing the same region of AIDA-1 proteins isolated from porcine AIDA-1 *E. coli* isolates PD58 and PD20. Identity = 12/12 (100%)

	825											836	
PD58	...	S	A	T	V	D	G	S	L	V	N	N	K	...
PD 20	...	S	A	T	V	D	G	S	L	V	N	N	K	...

Table 4 Coagglutination and immuno-dot-blot detection of AIDA-I protein on bacterial surfaces of porcine *E. coli* isolates determined to be positive or negative for AIDA-I gene by PCR.

AIDA-I positive *E.coli*			**AIDA-I negative *E.coli***		
Isolate	Coagglutination	Immuno-dot-blot	Isolate	Coagglutination	Immuno-dot-blot
PD20	++	++++	D123	+/-	-
PD22	++	++++	D126	+/-	-
PD23	+	+++	D127	-	-
PD58	+	++++	D132	+	-
PD74	+/-	++++	D136	-	-
PD75	+/-	++++	D140	-	-
PD80	++++	++++	D143	-	-
PD81	++++	++++	D144	-	-
PD100	++	-	D148	-	-
PD101	++	-	D152	-	-
PD102	+	-	D31	-	-
PD108	+	+	D59	-	-
PD124	+++	++	D73	-	-
PD134	+++	++++	D76	+/-	-
PD153	+	-	D82	+	+
PD80-1	+/-	++++	D83	++	++
PD80-2	+/-	++++	D96	-	-
			D99	+/-	-/+
			D35	-	-

Table 5 Experimental infection of neonatal piglets with wild AIDA-I *E. coli* strain PD20, its isogenic *aidA*⁻ mutant (PD20M) and *aidA*⁻ complemented strain (PD20C)

E. coli strain	Virulence factors	Inoculated (n)	Affected by diarrhea (n)	Onset of diarrhea (hr PI*)
PD20	AIDA-I⁺, STb⁺	8	8	20
PD20M	ΔAIDA-I⁻, STb⁺	7	0	n/a
PD20C	pT*aidA* (AIDA-I⁺, STb⁺)	6	6	30
PD71	Negative control	4	0	n/a

PI: Post inoculation

Table 6 Matched proteins in MASCOT database search for LCMS/MS peptide fragment mass fingerprints of p120 and p65

AIDA-I binding protein (MW)	Name of matched protein [species] in MASCOT database and NCBI access. #	Total MASCOT protein score	Predicted MW (kDa)	Predicted AA sequence of peptide	MASCOT peptide score	Significance of individual scores	
						Homology	Identity
p120 (120 kDa)	DNA damage binding protein 1 [*Homo sapiens*] gi|418316	287	128	LLASINSTVR	63	>49	>53
				YLAIAPPIIK	41	>48	>53
				IVVFQYSDGK	49	>37	>53
				VTLGTQPTVLR	44	>50	>53
				QSGESIDIITR	59	>46	>53
				EEQMDGTVTLK + Oxidation (M)	15	>28	>53
				IEVQDTSGGTTALRPSASTQALSSSVSSSK	19	>38	>49
	Splicing factor 3b [*Mus musculus* & *Homo sapiens*] gi|19527174 & gi|40254849	176	137	QQEIVVSR	30	>36	>54
				FLAVGLVDNTVR	76	>49	>53
				TVLDPVTGDLSDTR	73	>44	>52
	IgG Fc binding protein [*Homo sapiens*] gi|4503681	83	597*	GSQAVSYTR	42	>39	>52
				CFEGCECDDR	48	>43	>53
	IgG Fc binding protein [Rattus norvegicus] gi|34855405	53	608	GSQAVSYTR	42	>39	>52
				TDSFCPLQCPAHSHYSVCTR	17	>28	>50
p65 (65 kDa)	Cytokeratin 8 [*Mus musculus*] gi|13624315	85	54	YEDEINKR	28	>37	>53
				SLNNKFASFIDK	57	NA	>52
	Keratin 19 [*Gallus gallus*] gi|45384356	81	46	LASYLDK	42	>43	>53
				TTMQNLNDR + Oxidation (M)	40	>43	>53

Table 7 - Mutual relationship between fimbrial and non-fimbrial adhesins with toxin genes detected by multiplex PCR in 110 F4 negative *E. coli* strains isolated from diarrheic pigs.

Adhesin	No. of Strains	EAST1 STb	EAST1	EAST1 STb STa Stx2e	EAST1 STa Stx2e	STa	STb	LT STb	EAST1 STa	LT	EAST1 LT Sta Stx2e	EAST1 LT	Stx2e	Toxin negative
F4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F6	1	0	0	0	0	1	0	0	0	0	0	0	0	0
F18	1	0	0	1	0	0	0	0	0	0	0	0	0	0
F5, F41	1	0	0	0	0	1	0	0	0	0	0	0	0	0
F41	1	0	0	0	0	0	0	0	0	0	0	0	0	1
AIDA-I, F18	2	1	1	0	0	0	0	0	0	0	0	0	0	0
AIDA-I	7	7	0	0	0	0	0	0	0	0	0	0	0	0
EAE	4	0	1	0	0	0	0	0	0	0	1	1	0	1
Adhesin negative	93	1	16	0	1	1	2	1	1	1	0	0	1	68
Total	110	9	18	1	1	3	2	1	1	1	1	1	1	70

Table 8 - Clinical information and various virulence factors associated with 35 *E. coli* isolates of 18 diarrhea cases and co-pathogens.

Case #	Sample submitted	Isolate #	Adhesins							Toxins					Age (days)	Diarrhea	Co-infection	Morbidity (%)	Mortality (%)
			F4	F5	F6	F18	F41	EAE	AIDA-I	STa	STb	LT	Stx2e	EAST1					
1	ind	1	-	-	-	-	-	-	-	-	-	-	-	-	21-60	l, s	mixed *E. coli*	0-5	5-10
		2	-	-	-	+	-	-	-	+	+	-	+	+					
		3	-	-	-	-	-	-	+	-	+	-	-	+					
2	ind	4	-	-	-	-	-	-	-	-	-	-	-	-	21-60	l, sp	F4^{+} *E. coli*	5-10	1-25
3	ind	5	-	-	-	-	-	+	-	-	-	-	-	-	21-60	l, s	*Salmonella typhimurium*	0-5	5-10
4	ind	6	-	-	-	-	-	+	-	-	-	-	-	+	0-7	l, ps	*Clostridium perfringens*	25-50	1-5
		7	-	-	+	-	-	-	-	+	-	-	-	-					
5	ind	8	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, ps	-	5-10	1-5
6	ind	9	-	-	-	-	-	-	-	-	+	-	-	+	8-14	pw, ps	-	unknown	unknown
7	ind	10	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, p, ps	*Clostridium perfringens*	10-25	1-5
8	ind	11	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, r	-	25-50	1-5
9	ind	12	-	-	-	-	-	-	-	-	-	-	-	+	0-7	pw, sp	-	0-5	10-25
10	pool	13	-	-	-	-	-	-	+	-	+	-	-	+	21-60	pw, sp	*Salmonella typhimurium*, F4^{+} *E. coli* mixed *E. coli*	5-10	50-75
		14	-	-	-	-	-	-	-	-	-	-	-	+					
		15	-	-	-	-	-	-	+	-	+	-	-	+					
		16	-	-	-	-	-	-	+	-	+	-	-	+					

ind=individual sample; pool=pooled sample; pw= profuse watery; l=loose; p=pasty; ps=persistent; r=recurrent; s=sporadic; sp=single point of time

(continued)

Case #	Sample submitted	Isolate #	Adhesins							Toxins					Age (days)	Diarrhea	Co-infection	Morbidity	Mortality
			F4	F5	F6	F18	F41	EAE	AIDA-I	STa	STb	LT	Stx2e	EAST1					
11	ind	17	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, ps	*Clostridium perfringens*	10-25	1-5
		18	-	-	-	-	-	-	-	-	-	-	-	+					
12	ind	19	-	-	-	-	-	-	-	-	-	-	-	+	0-7	pw, ps	F4$^+$ *E. coli*	25-50	5-10
		20	-	-	-	-	-	-	-	-	-	-	-	+					
		21	-	-	-	-	-	-	-	-	-	-	-	+					
13	pool	22	-	-	-	-	-	-	+	-	+	-	-	+	21-60	l, sp	-	10-25	10-25
14	ind	23	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, r	mixed *E. coli*	5-10	1-5
		24	-	-	-	-	-	-	-	-	-	-	-	-					
		25	-	-	-	-	-	-	-	-	-	-	-	+					
		26	-	-	-	-	-	-	-	-	+	-	-	-					
		27	-	-	-	-	-	-	-	-	-	-	-	-					
15	ind	28	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, r	mixed *E. coli*	5-10	1-5
		29	-	-	-	-	-	-	-	-	-	-	-	-					
		30	-	-	-	-	-	-	-	-	-	-	-	+					
		31	-	-	-	-	-	-	-	-	-	-	+	+					
16	ind	32	-	-	-	-	-	-	-	-	-	-	-	-	8-14	l, r	mixed *E. coli*	5-10	unknown
		33	-	-	-	-	-	-	-	-	-	-	-	-					
17	ind	34	-	-	-	-	-	-	-	-	-	-	-	-	8-14	pw, l, ps	-	10-25	1-5
18	ind	35	-	-	-	-	-	-	-	-	-	+	-	-	8-14	pw, l, ps	-	10-25	1-5

ind=individual sample; pool=pooled sample; pw= profuse watery; l=loose; p=pasty; ps=persistent; r=recurrent; s=sporadic; sp=single point of time